EFFECT OF LONG-TERM EXPOSURE TO CADMIUM ON THE EARTHWORM *EISENIA FETIDA* (OLIGOCHAETA): POSSIBLE RESISTANCE

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Declaration

I, the undersigned hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entire or in part submitted it at any other university for a degree.

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Abstract

Environmental cadmium is the result of emissions from non-ferrous smelters and mining activities, as well as disposal of Cd-containing household waste Cd-containing sewage sludge and phosphate fertilisers. In polluted soil, the Cd associates with the biological fraction and therefore is relatively available for uptake by soil dwelling organisms

Animals living in contaminated soil are subjected to selection pressures caused by the pollutant and can either acclimate (the pressure is overcome by natural individual resistance), or develop a tolerance/adaptation (genetic increase in the mean resistance of the population). Numerous studies on soil invertebrates show that some species are capable of developing resistance to heavy metal pollution. For earthworms however, such data is non-existent.

The aim of this study was to determine whether a population of *Eisenia fetida* that had been exposed in the laboratory to sublethal concentrations of Cd for four years, had developed resistance to Cd.

This was done by subjecting pre-exposed and control worms to various experiments and comparing the results. The first parameter under investigation was biomass change of the different groups in uncontaminated, moderately contaminated, and heavily contaminated substrates. During these experiments, the pre-exposed worms displayed signs of Cd-dependency by performing worse in the uncontaminated substrate than in the moderately contaminated substrate. This was also reflected when cocoon production was monitored, the control worms showing a linear decrease in cocoon production as the concentration of $CdSO_4$ in the substrate rose, while the pre-exposed worms had the highest cocoon production in the moderately contaminated substrate.

Physiologically, the osmolality- as well as the cell percentage of the coelomic fluid for pre-exposed and control worms, exposed to high concentrations of $CdSO_4$ was measured. Although no differences between the two groups were observed when looking at the osmolality, a possible resistance was observed for the pre-exposed worms when looking at the differences in cell percentage.

Total body accumulation did not differ between the pre-exposed and control worms after exposure to Cd. The pre-exposed worms did however show a marked increase in the nephridial accumulation of Cd as shown by PIXE analyses of the organs, when compared to the control groups.

This study has shown that there is definite evidence of an increased resistance to Cd in the form of $CdSO_4$ in the pre-exposed worms when compared to unexposed control worms. Whether this resistance is the result of acclimation or a genetic adaptation could however not be determined by this study and further research in that direction is recommended.

Opsomming

Kadmium beland in die omgewing deur emissies afkomstig van nie-fereuse smelterye en mynaktiwiwteite, asook deur die kadmiumbevattende huishoudelike afval, riool en fosfaatkunsmisse. In die besoedelde grond assosieer die kadmium met die biologiese fraksie en is dus relatief beskikbaar vir opname deur grondlewende organismes.

Diere in besoedelde grond word blootgestel aan seleksiedruk. Diegene wat kan oorleef, het òf geakklimeer (oorkoming deur die individu se natuurlike weerstand), òf geadapteer ('n genetiese toename in die gemiddelde weerstand van die populasie). Verskeie studies is gedoen op grondinvertebrate wat aantoon dat sekere spesies wel 'n verhoogde weerstand ten opsigte van swaarmetaalbesoedeling kan ontwikkel. Baie min inligting is egter beskikbaar oor erdwurms in hierdie verband.

Die doel van hierdie studie was om vas te stel of 'n populasie van *Eisenia fetida,* wat oor 'n tydperk van vier jaar in die laboratorium blootgestel is aan subletale konsentrasies van Cd, 'n verhoogde weerstand ten opsigte van Cd toon. Dit is gedoen deur voorafblootgestelde en kontrole wurms aan verskeie eksperimente te onderwerp en die resultate te vergelyk. Die eerste parameter waarna gekyk is, is die verandering in biomassa van die ondersreie groepe in 'n ongekontamineerde ,matige of swaar gekontamineerde substraat. Hierdie eksperimente het getoon dat die voorafblootgestelde wurms tekens van Cd-afhanklikheid toon deurdat hulle swakker gevaar het in die ongekontamineerde as in die matig gekontamineerde substraat. Tekens van 'n Cd-afhanklikheid is ook waargeneem toe daar na kokonproduksie gekyk is. Die kontrolewurms het 'n liniêre afname in die kokonproduksie getoon namate die konsentrasie van die Cd in die substraat toegeneem het, terwyl die

voorafblootgestelde wurms die hoogste kokonproduksie getoon het in die matig gekontamineerde substraat. Fisiologies is daar gekyk na die osmolaliteit en die selpersentasie van die seloomvloeistof van die voorafblootgestelde en kontrole wurms by blootstelling aan hoë konsentrasies van Cd. Alhoewel daar geen verskille tussen die twee groepe waargeneem was, wat osmolaliteit betref nie, was daar wel aanduidings van 'n verhoogde weerstand gevind in die voorafblootgestelde wurms toe die selpersentasies ondersoek is.

Die totale liggaamskonsentrasie van Cd het nie verskil tussen die voorafblootgestelde en kontrole wurms na blootstelling aan Cd nie. Die voorafblootgestelde wurms het egter 'n duidelike toename in nefridiale akkumulasie van Cd getoon na PIXE analise van dié organe.

Hierdie studie het aangetoon dat daar 'n definitiewe aanduiding is van 'n verhoogde weerstand t.o.v. Cd in die voorafblootgestelde wurms. Of hierdie weerstand die gevolg van akklimasie of geneties ontwikkelde toleransie (weerstand) is, kon egter nie deur hierdie studie aangetoon word nie, en verdere navorsing op hierdie gebied word voorgestel.

Dedication

I would like to thank my parents who have supported me throughout my pre- and post-graduate studies. Although, as a child, one does not always show appreciation for the sacrifices that parents make for you, be assured that it has been recognised and appreciated.

For their continued support and encouragement, I would like to dedicate this thesis to my father, Jacob George Prinsloo and mother, Martha Louise Alma Prinsloo.

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Contents

CHAPTER 1

General introduction

Cadmium is a relatively rare heavy metal occurring naturally with zinc. It is refined as a by-product of zinc smelting. Most cadmium occurring in the soil ecosystems are there as a result of human activity. Since 1990 the world refinery production of Cd has stabilised from 20 200 metric tons annually in 1990 (Kuck, 1994) to 19 900 metric tons annually in 1998 (Palchy, 1999). Estimates of recycling for Cd are currently unknown (Palchy, 1999) but nickel-cadmium batteries are the major source of recycled Cd. Most Cd-containing products are dumped together with household waste, thereby contributing to the contamination of the environment. This problem is worsened when the waste is burned, as the Cd-containing dust is distributed over a wider area. Cd emissions into the environment from non-ferrous smelters and mining activity were estimated to be about 7000 metric tons annually in 1988 (Nriagu & Pacyna). A proportion of all the Cd emitted into the atmosphere will eventually be deposited on the soil, with additional inputs from the use of Cd-containing sewage sludge and phosphate fertilisers (Elinder & Järup, 1996). Flue dust, originating from smelters has also been applied in some cases to raise the soil pH (Li & Shuman, 1996).

Earthworms, living in the soil environment, are directly at risk from Cd pollution. In the soil, Cd binds preferably to the organic fraction (Li & Shuman, 1996), thus making it more available to the worms. The decrease in pH caused by acid rain and other factors, may also increase the toxicity of Cd to the worms (Bengtsson,

Gunnarsson & Rundgen, 1986) by increasing the mobility and absorption of the heavy metal.

The occurrence of terrestrial invertebrates in polluted soils raises the question whether or not these animals can evolve a resistance to heavy metals by means of adaptation (tolerance) or acclimation. Heavy metal adaptations in terrestrial invertebrates have been reported in numerous cases, as reviewed by Posthuma and Van Straalen (1993). Most studies on invertebrate adaptations are however concentrated in the field of pesticide tolerance in insects, mainly for economic reasons (Georghiou, 1986). One implication of a genetic resistance of a population, or acclimation of individuals, for a scientific study, is that the results obtained from using these animals in toxicological tests may be biased.

According to Posthuma and Van Straalen (1993), the following considerations should be kept in mind when doing studies on metal-adaptation:

- Local adaptations may indicate the presence of a toxic effect at the site.
- Studies on adaptation show that there is a presence or absence of genetic variability of tolerance and other features within the population. This is important, since genetic variability determines the potential for a response to future environmental stress factors, originating either from other pollution events, or from natural factors.
- Studies of tolerance can indicate critical physiological pathways that are involved in the tolerance.
- These studies may identify the consequences of strong directional selection for fitness, also known as the "cost of tolerance": Heavy metal adaptation is expected to cause a reduction of fitness, which is shown by non-tolerant characteristics, when compared to a reference group.

An increased metal tolerance can arise from exposure of an individual (acclimation), or from natural selection (adaptation), or both (Posthuma & Van Straalen, 1993). Physiological acclimation implies that the individual being studied has acquired some degree of tolerance after pre-exposure to a sublethal concentration during some period of its life. This tolerance is not genetic, and thus not passed on to the individual's offspring. Acclimation can therefore be seen as a type of phenotypic plasticity and thus can be induced and lost within one generation. A genetic adaptation however, implies that the population has evolved an increase in its mean tolerance through natural selection.

Genetic adaptation occurs by means of the process called natural selection, which operates on a genetically based inter-individual variation in tolerance. This is the process whereby some alleles are retained while others are eliminated. When this occurs in a polluted environment under the influence of toxic substances, genotypes that are eliminated are called "susceptible", and those that are retained, "resistant" (Holloway, Silby & Povey, 1990). According to Holloway *et al.* (1990) the ability of an organism to resist a toxin, may be physiologically expensive in terms of energy and other resources. The resistance may therefore, out of necessity, involve a diminution of the ability to invest energy in other processes.

One form of such compensationary mechanisms developing as a result of natural selection, is the r- and K-strategies mentioned by Satchel (1980). The author suggests that r and K selection could explain the differences in behaviour, morphology and physiology of the endogeic (deep dwelling) and epigeic (surface dwelling) lumbricid earthworms:

- The r-strategy evolves by selection for worms that allocate most of their energy into reproduction. These worms have a short life span, and a high reproductive rate.
- For the K-strategy on the other hand, survival of the individual is placed above reproduction. These worms would thus have a long life span, and a low reproductive rate.

Animals under toxicological stress can also employ such an r- or K-strategy as a strategy for survival. This may be reflected in life history parameters, such as maturation age, cocoon production, cocoon viability, etc.

Heavy metal resistance has been shown to occur in various terrestrial invertebrates, which include the Protozoa, some Oligochaeta, Mollusca, Crustacea, Myriapoda, Arachnida, Apterygota, Orthoptera, Hemiptera, Lepidoptera, Diptera and Coleoptera, as reviewed by Posthuma and Van Straalen (1993). Broeks, Gerrard, Allikmets, Dean & Plasterk, (1996) found that the soil nematode *Caenorhabditis elegans* also developed a heavy metal tolerance, which could be attributed by gene homologues to the human multidrug resistance genes. A Cd-tolerance has been shown for the marine oligochaete *Limnodrillus hoffmeisteri* (Klerks & Bartholomew, 1991) inhabiting the heavily polluted Foundry Cove situated on the Hudson River (NY). Klerks and Levinton (1989) showed that these worms could have evolved the metal tolerance within 1 to 4 generations. A study conducted on the evolutionary response of the earthworm *Dendrobaena octaedra* to long-term metal exposure, 150-200m removed from a brass mill (Bengtsson, Ek & Rundgen, 1992), however, did not show any conclusive evidence that the heavy metals (Zn, Cu and Cd) in the soil excerted any significant selection pressure on *D. octaedra.*

The question raised in this thesis is whether or not the earthworm *Eisenia fetida* could develop a resistance to Cd after long-term exposure to this xenobiotic heavy metal. Reinecke, Prinsloo & Reinecke, (1999) have shown that there is an indication of resistance developing in *E. fetida* after long term exposure. The present study aims to explore possible differences between a long term exposed group of *E. fetida*, and an unexposed control group, to determine whether any indications of a possible resistance, are present in pre-exposed animals.

As stated by Holloway *et al.* (1990), the ability of an organism to resist a toxin may be physiologically expensive, resulting in a diminution of the ability to invest energy into other processes. Therefore, two groups of clitellate worms of the species *E. fetida* that originated from 1) a culture in which they received exposure to Cd in the form of $CdSO_4$ for four years, and 2) an uncontaminated control culture, were investigated to detect possible indications of an increased resistance in the pre-exposed group. For this, life history parameters were investigated as well as the effects of Cd on the excretory system, which in vertebrates is the critical system for Cd accumulation and poisoning (Elinder, 1977; Roels, Djubgang, Buchet, Bernard & Lauwerys,1982; Elinder & Järup, 1996; Leffler & Nyholm, 1996; Lind, Engman, Jorhem & Glynn, 1998; and Reeves & Vanderpool, 1998).

Life history parameters investigated include:

a) Monitoring changes in biomass between the pre-exposed and control group in substrates contaminated with different concentrations of $CdSO_4$.
b) Determining the cocoon production in these different exposure conditions.
c) The amount of viable cocoons produced by each group.
d) The amount of worms hatching from each viable cocoon.

e) The concentration of Cd accumulated in the bodies of the experimental worms throughout the duration of the experiment.

Physiological/morphological parameters investigated include:

a) Histological evaluation of the nephridia after exposure to high concentrations of $CdSO_4$.

b) Possible differences in the osmolality of the coelomic fluid after exposure to high concentrations of $CdSO_4$.

c) Possible differences in the amount of coelomocytes present in the coelomic fluid after exposure to high concentrations of $CdSO_4$.

d) Nephridial accumulation of Cd after exposure to high concentrations of $CdSO_4$.

CHAPTER 2

General materials and methods

2.1 *Eisenia fetida*

2.1.1 Classification

Phylum: Annelida

Class: Oligochaeta

Suborder: Lumbricina

Superfamily: Lumbricoidae

Family: Lumbricidae (Rafinesque-Schmaltz, 1815)

Subfamily: Lumbricinae (Rafinesque-Schmaltz, 1815)

Genus: *Eisenia* (Malm, 1877)

Species: *fetida* (Savigny, 1826)

Subspecies: *fetida* (Bouché, 1972)

2.1.2 Morphology

E. fetida has an approximate length between 35 and 130 mm, but usually less than 70mm. Its body is cylindrical with closely paired setae. The body comprises of about 20 – 110 segments, with a clitellum (6 to 8 segments in length) developing from segment 24/25 or 25/26 to segment 31/32 or 32/33. Tuberculae puberatis are found from segment 27 to 30 (Gates, 1956; Reynolds, 1977). Colour varies from purple to brownish red. Sometimes alternating bands of red-brown occur on the dorsal surface, with pigmentless yellow intersegmental areas (Reynolds, 1977)

Large glandular papillae, together with the male pores are situated on segment 15. Four pairs of seminal vesicles occur in segments 9 to 12, and 2 pairs of spermathecae with their spermatic ducts opening in the midline between segments 9/10 and 10/11 (Reynolds, 1977).

2.1.3 Life Cycle

The lifecycle of *E. fetida* is relatively short, the animals reaching maturity within 7 to 8 weeks after hatching at temperatures of 15-20°C (Edwards & Bohlen, 1992). A schematic representation of the life cycle is given in Figure 2.1.

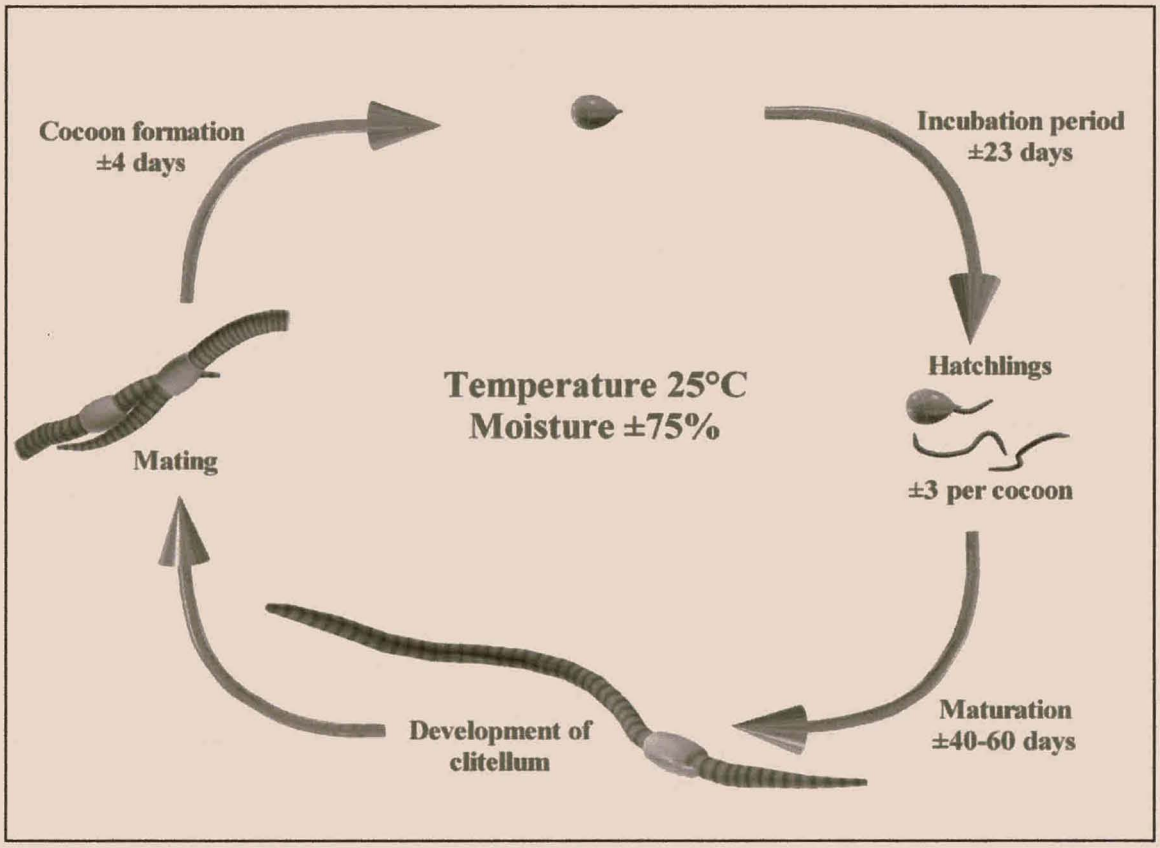

Figure 2.1 Schematic representation of the life cycle of *Eisenia fetida* (adapted from Venter & Reinecke 1988)

Mating occurs throughout the year, except when the climatic conditions are unfavourable or the earthworms are in diapause (Reynolds, 1977). Cocoon production

begins within four days of mating (Venter & Reinecke, 1988). Each worm may produce 2 to 5 cocoons per week (Edwards & Bohlen, 1992), with each cocoon normally producing more then one hatchling (Reinecke & Kriel, 1981a).

Fecundity is dependent on soil humidity, temperature and the availability of food (Reinecke & Kriel, 1981a). The worms have a life expectancy of four to five years, with an average of about 1-2 years (Reynolds, 1977; Satchell, 1967)

2.1.4 Distribution

E. fetida is a native palaerctic, occurring over the greater part of Europe, North America and Russia (Reynolds, 1977). Due to the distribution of the species by man, where they have in some cases replaced the indigenous earthworm fauna (Satchell, 1967), they can be considered as a cosmopolitan species, occurring also in South America, Asia, Africa, Australasia and Iceland (Reynolds, 1977). *E. fetida* does not occur in large numbers in the soil (Edwards & Bohlen, 1992) but is normally found in areas where there are large quantities of decaying matter, such as compost heaps and manure piles (Satchell, 1980; Reinecke & Kriel, 1981b; Reynolds, 1977), with a substrate pH of 6.8 to 7.6 (Reynolds, 1977). In South Africa *E. fetida* is commonly found in compost heaps, and is used for vermicomposting.

2.2. Cadmium:

2.2.1 History, production and uses of cadmium:

Cadmium (Cd) is the sixty-seventh most abundant element in the earth's crust. It occurs as isomorphic impurities in, or surface coatings of other sulfide minerals, especially the zinc sulfides, where it can reach concentrations as high as 5%. F. Strohmeyer, a professor of metallurgy at Göttingen, Germany, discovered it in 1817. In 1819 W.Meissner and C.J.B. Karsten confirmed the new element (Nriagu, 1980a).

It is primarily recovered entirely as a by-product from residues obtained during the smelting of zinc, lead, zinc-lead, zinc-copper, and complex ores. The supply of cadmium thus depends largely on the activities in the zinc industry and to a lesser extent, the lead and copper industries.

Production began in earnest after 1871, when cadmium and its compounds were used as paint pigments and for other purposes. Before 1971 production did not exceed 100kg annually. In 1871 and 1872 production was 710 and 1818 kg respectively, but by 1910 annual production had exceeded 43 000 kg (Chizhikov, 1966)

2.2.2 Consumption:

The first major application of Cd was in paint pigments. This occurred more than 60 years after the element was discovered. Other early applications of cadmium included low-melting alloys, electroplating, glass making, photography, as salts in dentistry, dying, calico printing and as chemical reagents (Nriagu, 1980a). From 1966-1975 50% of Cd consumption in the USA was accounted for by electroplating. Plastic stabilisers and batteries combined represented about 40% of the US cadmium

consumption. A breakdown of the Cd consumption in the western world in 1997 was as follows: batteries 70%, pigments 13%, coatings and plating 8%, stabilisers for plastics and similar synthetic products 7%, with alloys and other uses making up the remaining 2%. The total world refinery production of Cd in 1997 was estimated at about 18 9000 metric tons (Palchy, 1997)

2.2.3 Global cadmium cycle

There are active pools in the environment, which are subject to large inputs of Cd from pollutant sources. These active pools (reservoirs) include the atmosphere, soils, lakes, rivers and the ocean. The exchange of Cd between reservoirs usually occurs among established routes (Fig. 2.2), involving streams, ice- and groundwater flows, atmospheric transport and deposition, volcanism, uplift, subaqueous weathering, sedimentation, and also various biological pumps. The world wide cadmium input to land areas from atmospheric fallout and waste disposal plus fertiliser applications were estimated to be 5.7×10^9 and 1×10^9 g/year respectively in 1980 (Nriagu, 1980 b).

Humans have become a major macrobiological agent in the biochemical cycle of cadmium (Nriagu, 1980b). It was estimated that by 1980 the amount of Cd released into the atmosphere since it's discovery in 1817, was around 320×10^9 g. In addition to this, a further 500×10^9 g had been produced and dissipated on the earth's surface. This redistribution of Cd affects the concentration fluxes of the metal in many ecosystems. This can be seen in the arctic snowfields, where a 2- to 10-fold increase in the atmospheric deposition of Cd over the past 300 years, has been recorded (Weiss, Bertine, Koide, & Goldberg, 1975).

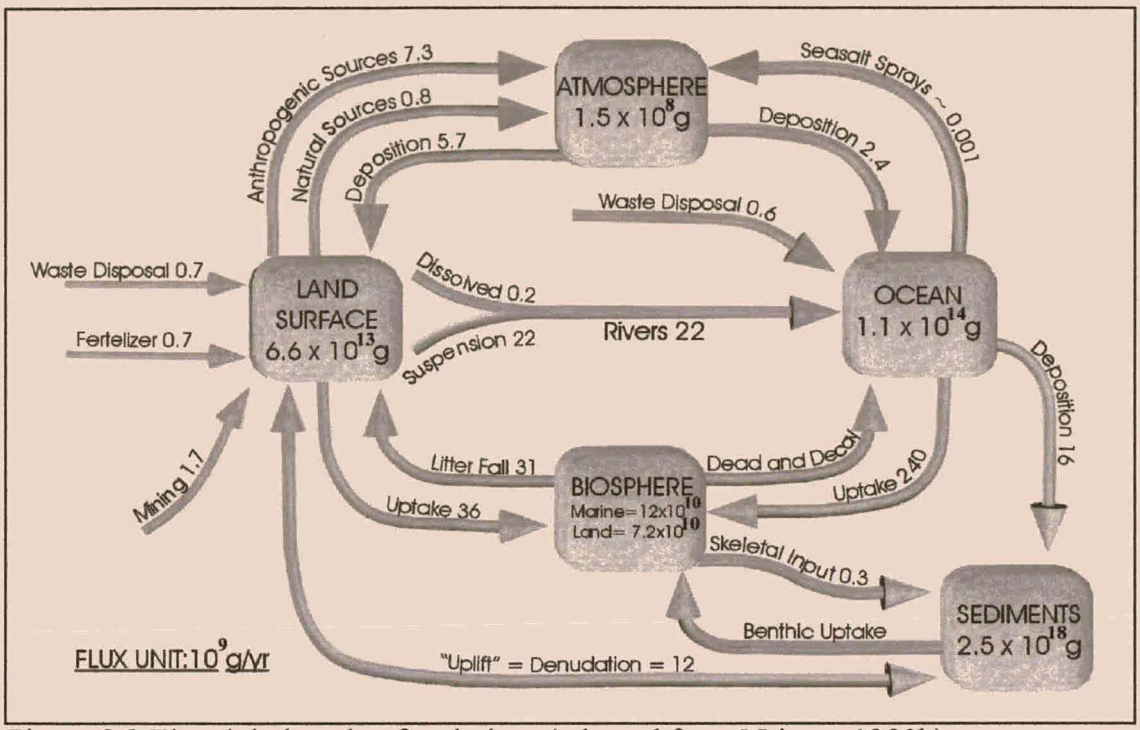

Figure 2.2 The global cycle of cadmium (adapted from Nriagu, 1980b)

2.3 Experimental Materials and methods

2.3.1 Rearing:

Stock cultures of *E. fetida* were maintained in the laboratory at a constant temperature of 25°C and 70% humidity, the substrate consisted of ground and sieved cattle manure. These cultures were fed on a weekly basis with fresh urine-free cattle manure.

Two cultures were used for this study. The first was a control culture. Worms originating from this culture had no exposure to Cd for four years. In the text, worms originating from this culture will be referred to as unexposed (Cd_{ABS}-) worms ($_{ABS}$ = absent). The second culture was contaminated with Cd over four years, by the weekly addition of 0.01% $CdSO_4$ to their food. Since the average life cycle of *E. fetida* is

about three to four months (Venter & Reinecke, 1988), at least 10 generations have been exposed to Cd in this culture. Worms originating from this culture will be referred to in the text as pre-exposed (Cd_{PRE}-) worms ($_{PRE}$ = pre-exposed).

2.3.2 Artificial soil substrate

Fourteen-day exposure experiments, to determine the effects of Cd on the nephridia and water homeostasis, were conducted in an artificial soil substrate. This substrate was prepared using the OECD guideline no.207 (OECD 1984) with the artificial soil consisting of 70% silica sand, 20% kaolin clay and 10% peat moss. These were mixed thoroughly after which the pH of the mixture was adjusted to 6.0 ± 0.5 by the addition of chemically pure $CaCO_3$ powder (ASSOCIATED CHEMICAL ENTERPRISES $_{(PTY)\ LTD}$). The silica sand was obtained from the region of Kraaifontein in an open field at a depth of about 1.5 m. Before use, the sand was rinsed at least 10 times until the water was clear after rinsing. The sand was then dried at 70°C and sieved to a particular size < 500 µm. The kaolin clay was obtained from T.REINDERS™ Potters Supplies (Kraaifontein), and the peat moss used was SHAMROCK™ Irish peat moss, obtained from the Stodels nursery in Durbanville.

One-litre plastic containers were used for the fourteen-day exposure experiments. Each container was filled with 500 g dry artificial soil, after which the test substance ($CdSO_4$), diluted in distilled water, was added to give a moisture content of about 35%. All the ingredients were mixed by hand, and the wetted substrate was then placed in a climate-controlled room with a temperature of 25°C and a relative humidity of 70%, for at least 24 hours to stabilise before worms were introduced.

2.3.3 Cattle manure substrate

For the experiments in which the life history parameters were investigated (8 weeks), a cattle manure substrate, as described by Reinecke and Venter (1987) was used. Urine-free cattle manure was air-dried, ground and sieved to a particle size between 1000 and 500 µm. This substrate was then wetted by distilled water to a moisture content of 75%. The wetted substrate was placed in a climate-controlled room with a temperature around 25°C and a relative humidity of 70%, for at least 24 hours to stabilise before worms were introduced.

2.3.4 Containers

For all experiments plastic containers were used. The volume of the containers used for the experiments was one litre. The lids were perforated and covered with fine gauze to prevent the escape of the worms, but still allow aeration. During the course of the experiments, the containers were placed in a climate-controlled room, with a constant temperature around 25°C, and a humidity of 70%.

2.3.5 Acid digestion

Worms from all experiments that were not used for histological or PIXE (proton induced X-ray emission) analyses, were acid digested to determine the amount of Cd accumulated in the worms. Worms destined for acid digestion were placed on filter paper for 24 hours to depurate the gut, where after they were killed by freezing. These worms were then dried for 48 hours at 70°C, weighed and acid digested.

Samples of the substrates used in the experiments were also dried for 48 hours at 70°C, and then acid digested to determine the dry weight concentration of Cd in these

substrates. Earthworm and substrate samples were digested using the following procedure:

1. 10 ml Nitric acid was added to each sample and the samples then left over night. The following day the samples were heated for two hours at a temperature of 40°C, and then for another hour at a temperature between 120 and 135°C (Katz & Jeniss, 1983). If easily oxidized substances come into contact with perchloric acid, this may result in a vigorous reaction. Therefore the samples were first boiled in concentrated nitric acid before the addition of perchloric acid (Feldman, 1974). The solution resulting from this digestion usually had a brown to tan hue.

2. After the initial boiling in nitric acid, the samples were allowed to cool, upon which 1 ml perchloric acid was added to the earthworm samples, and 5 ml to the substrate samples. The samples were then heated for another hour at a temperature between 125 and 135°C (Katz & Jeniss, 1983). Depending on the organic content of the original sample, the resulting solutions usually had a pale yellow hue.

3. The samples were then again allowed to cool down, after which 5 ml distilled water was added to each sample, which were then heated until white fumes were given off.

After the digestion process, the samples were centrifuged at 3000 rmp for 5 minutes and only the supernatant removed for filtering. The supernatant samples were filtered twice, first using a No.6 Whatman and then a 0.45 µm Millipore filter, and made up to 20 ml using distilled water.

Adsorption of the metals on to glassware may give rise to errors. This is a particular problem when the trace element concentrations in the solution are very low (less than $10mg.l^{-1}$). To prevent this from happening, the samples were transferred to plastic containers for storage directly after making them up to 20 ml (Ebdon, 1982). Errors may also occur due to contamination from airborne particles and laboratory ware. To overcome this problem, blanks were run during each batch of samples digested (Ebdon, 1982; Hopkin, 1989).

Analyses of the digested samples were conducted using a Varian AA-1275 flame atomic absorption spectrophotometer at the department of Physics (Stellenbosch University), who also supplied the standards for calibration.

2.3.6 Statistical analyses

All numerical data obtained for the different experiments were analysed for homogeneity of variance using Bartlett's test. When the variance was homogenous among the groups, one-way analyses of variance (ANOVA) were carried out. If significant differences were found between the groups using an ANOVA, a Dunnett test was run to determine which groups differed from one another by comparison of the mean values of each group. Where the variance was found to be heterogeneous based on the Bartlett test, a Kruskal-Wallis H-test was used to look for differences among the groups. If significant differences were found, the mean values of the groups were compared to one another by means of the Student-Newman-Keuls method. Statistical significance was concluded at the 5% level ($P = 0.05$). These statistical tests were executed with Sigma Stat (Jandel Scientific) ver.2 for Windows.

CHAPTER 3

Effect of $CdSO_4$ on life history parameters

3.1 Introduction

Numerous species of terrestrial invertebrates have shown adaptation (Posthuma & Van Straalen, 1993). For earthworms, however, conclusive evidence to demonstrate a response to selection is absent. This lack of evidence for adaptation in terrestrial annelids is contrasted by the occurrence of adaptation in the aquatic oligochaete *Limnodrilus hofmeisteri* inhabiting the Cd-, Ni- and Co-polluted Foundry Cove on the Hudson river, NY (Klerks & Levinton, 1989). Morgan and Morgan (1988) have observed differences in accumulation patterns for Cd and Zn, as well as sensitivity divergence for Pb, during field transplant experiments with field captured *Lumbricus rubellus*. Morgan, Norey, Morgan & Kay, (1989) also demonstrated that *L. rubellus* and *Dendrodrilus rubidus* originating from a polluted site were able to synthesise Cd-binding proteins. Suzuki, Yamamura & Mori (1980), as well as Yamamura, Ishiguro, Saito & Suzuki, (1981) demonstrated that *E. fetida* has a phenotypic plasticity for the production of metallothionein, which means that there is an increase in metallothionein production upon exposure.

On the level of life-history parameters, selection for tolerance is expected to improve fitness in exposed conditions. This would be expressed by an improved performance of these characteristics when measured in exposed conditions, as opposed to exposed sensitive conspecifics. The evolutionary response (tolerance) is

the direct cause of improvements to help the organism cope in the polluted environment, and thus fitness characteristics are indirectly affected. Mechanisms underlying tolerance involve metal-binding proteins, altered accumulation patterns, conservation of energy and other resources, increased excretion or immobilisation in granulae, and possibly alleles coding for protein with decreased sensitivity for metal ions within the cell (Posthuma & Van Straalen, 1993).

According to Posthuma & Van Straalen (1993) the theory of life-history is concerned with the evolutionary responses to environmental factors that affect life-history characteristics, such as development time and clutch size. It is usually restricted to selection pressure from natural origin, rather than that of pollutants. However, responses to life-history characteristics can be expected from any factor that affects age-specific survival or reproduction (Michod, 1979) including pollutants. A pollutant that causes a reduction in adult survival and reproductive allocation, is expected to induce an earlier maturation and an increased allocation of energy to reproduction in animals living in the polluted environment. The opposite is true for an increased adult survival and reproductive allocation. In the soil polluted by heavy metals, exposure is chronic and toxic effects usually consist of an impairment of growth and reproduction, as well as mortality (Bengtsson, Gunnarsson & Rundgen, 1983). Since the toxic effects of heavy metals are likely to increase with the age of the exposed animals - metals accumulate at a rate comparable to the development rate (Janssen, Bruins, De Vries & Van Straalen, 1991) - it is expected that populations that are exposed to Heavy metal pollution will have an evolutionary response to this chronic exposure. This evolutionary response usually takes the form of an earlier maturation and an increased allocation of energy to reproduction. This prediction is only valid however, if the population can achieve these modifications in the

conditions of the polluted environment. The allocation of energy to tolerance mechanisms and other functions is of central importance (Sibly & Calow, 1989), and may place constraints on the array of possible responses towards the pollutant. It should be remembered though that for any evolutionary process to develop, it is crucial that a genetic variance for the tolerance mechanism should be present.

Various life-history changes have been observed in pollution exposed populations, probably due to the constraints of genetic variation. It has been demonstrated that some metal-tolerant populations of plants exhibit a decreased metabolic rate and slow growth, in order to preserve energy and/or other resources (Antonovics, Bradshaw & Turner, 1971). This may also apply to the tolerant isopods studied by Donker (1992).

For this study, the following life-history parameters were studied, to determine whether or not a population of *E. fetida* that received chronic exposure to Cd in the form of $CdSO_4$ for more than 10 generations, had developed signs indicative of a Cd-resistance when compared to a control population. The first life-history parameter monitored was the change in biomass displayed by clitellate worms of the two groups placed in different contaminated substrates, followed by the cocoon production of the two groups, as well as the percentage of viable cocoons, and the amount of hatchlings per viable cocoon. At the end of the exposure period, the concentration of Cd accumulated within the bodies of the different groups was also determined to assist in explaining changes in life history parameters where they occurred.

3.2 Materials and methods

3.2.1 Exposure

Worms originating form the long-term exposure culture (Cd_{PRE}-worms), as well as worms originating from the control culture (Cd_{ABS}-worms) were exposed to 0, 600 and 1200 $mg.kg^{-1}$ $CdSO_4$ in experimental cattle manure substrates (See section 2.3.3.) for the duration of eight weeks.

At the beginning of the experiment a stock supply of 1 kg cattle manure substrate was prepared for each experimental group. Three groups of both Cd_{ABS}- and Cd_{PRE}-worms, each consisting of twenty clitellate worms, were exposed to the respective substrates. This was done by placing the worms in 400 g of the respective substrates. The worms were then kept in a climate-controlled room at a temperature of 25°C and relative humidity of 70%. Each week the worms were weighed, and the cocoons sorted out by hand. In a similar experiment conducted by Reinecke *et al.* (1999) it was shown that 400g of cattle manure substrate resulted in an increase in mean body weight of the worms for three weeks, whereafter a weight decrease was observed. From this it can be deduced that 400 g cattle manure substrate contained sufficient food for the duration of three weeks. In this experiment therefore, the substrate to which the worms were exposed, was replaced by another 400 g of substrate from the respective stock substrates after the third week of exposure, and again after the sixth week with 300 g. This was done to assure that the worms had sufficient food for the eight-week duration of the experiment.

Cd_{ABS}-worms =Worms originating from a culture where they received no exposure to any heavy metals over a period of four years

Cd_{PRE}-worms =Worms originating from a culture where Cd had been administered over a period of four years

Cd_K-worms =Control group, consisting of worms originating from the same culture as the Cd_{ABS}-worms, but that received no exposure during the course of the experiment.

3.2.2 Hatching success and number of hatchlings per cocoon

The cocoons that were hand sorted from the experimental substrates after each week, were placed in multicell containers (Corning®, disposable multiple well plates, 25820-24) which were filled with uncontaminated cattle manure substrate to provide a more natural incubation environment, and to ensure that the hatchlings had food at their disposal. The cocoons were then placed in a growth cabinet at a temperature of 25°C, hatching success and the amounts of worms originating from each viable cocoon was monitored twice a week. Since the hatching time for *E. fetida* is about 23 days (Venter & Reinecke, 1988), the cocoons were monitored for 28 days. Cocoons that had not hatched by that time were assumed to be sterile.

3.2.3 Body accumulation of Cd

After the eight-week exposure period, six worms from each of the six experimental groups were removed to determine the total body burden of Cd. This was done by means of acid digestion and flame atomic absorption spectrometry as described in section 2.3.5.

3.3 Results

The percentage change in biomass between the Cd_{ABS}- and Cd_{PRE}-worms placed in clean, uncontaminated cattle manure substrate is represented in Figure 3.1. It was found that the Cd_{ABS}-group maintained a positive change in biomass throughout the eight weeks. The Cd_{PRE}-group however, experienced a positive change until the fourth week whereafter the group experienced a negative change in biomass. An ANOVA was carried out ($H = 252.86$ with 15 degrees of freedom, $P < 0.001$). At weeks 2, 4 and 6, no statistically significant differences were observed between the Cd_{ABS} and Cd_{PRE}-groups ($P > 0.001$)

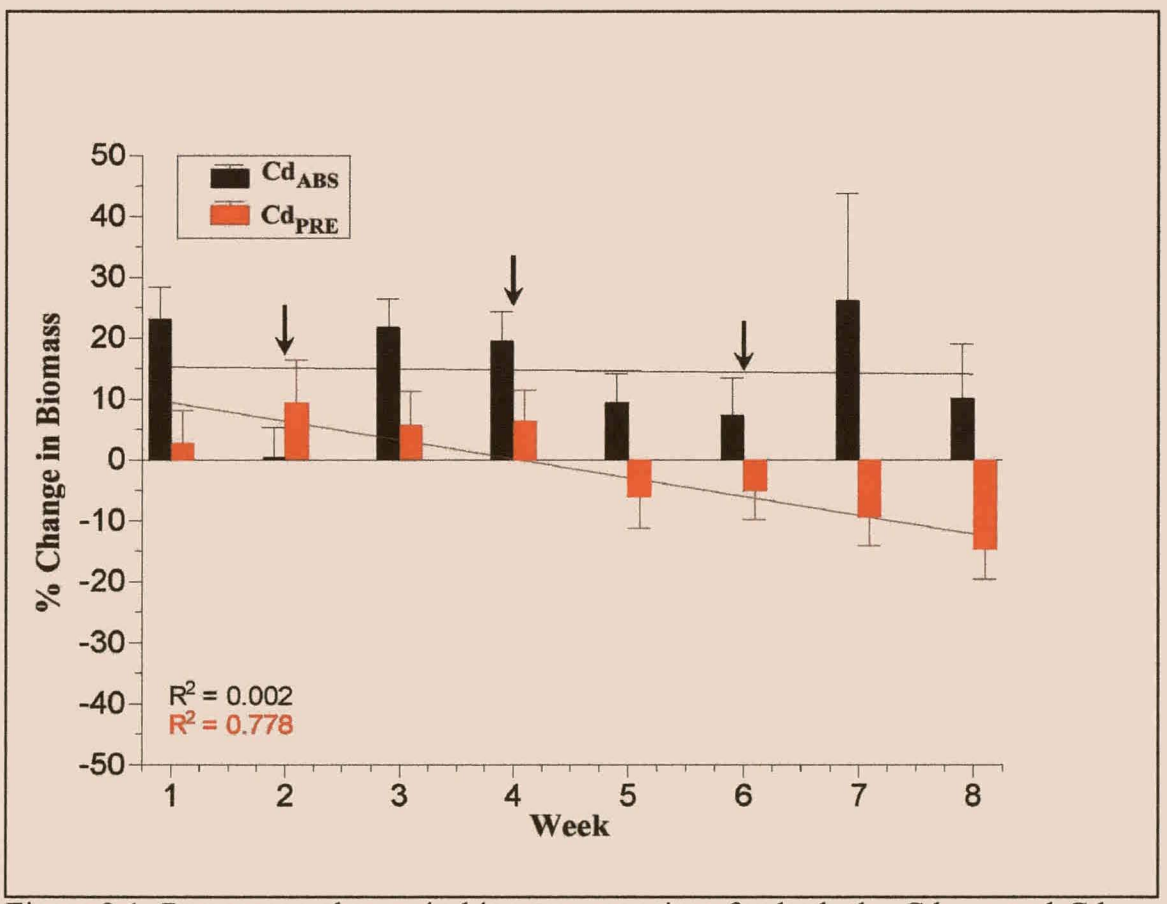

Figure 3.1 Percentage change in biomass over time for both the Cd_{ABS}- and Cd_{PRE}-worms in an uncontaminated cattle manure substrate. The arrows indicate the weeks where the Cd_{ABS}- and Cd_{PRE}-groups showed no statistically significant difference ($P > 0.001$).

In the substrates contaminated with 600 $mg.kg^{-1}$ $CdSO_4$, both the Cd_{ABS}- and Cd_{PRE}-worms showed a positive change in biomass (Figure 3.2). In the first, third and last two weeks, no statistically significant difference was found in the mean change in biomass between the two groups. An ANOVA was carried out ($H = 195.129$ with 15 degrees of freedom, $P < 0.001$). At weeks 1, 3, 7 and 8, no statistically significant differences were observed between the Cd_{ABS} and Cd_{PRE}-groups ($P > 0.001$)

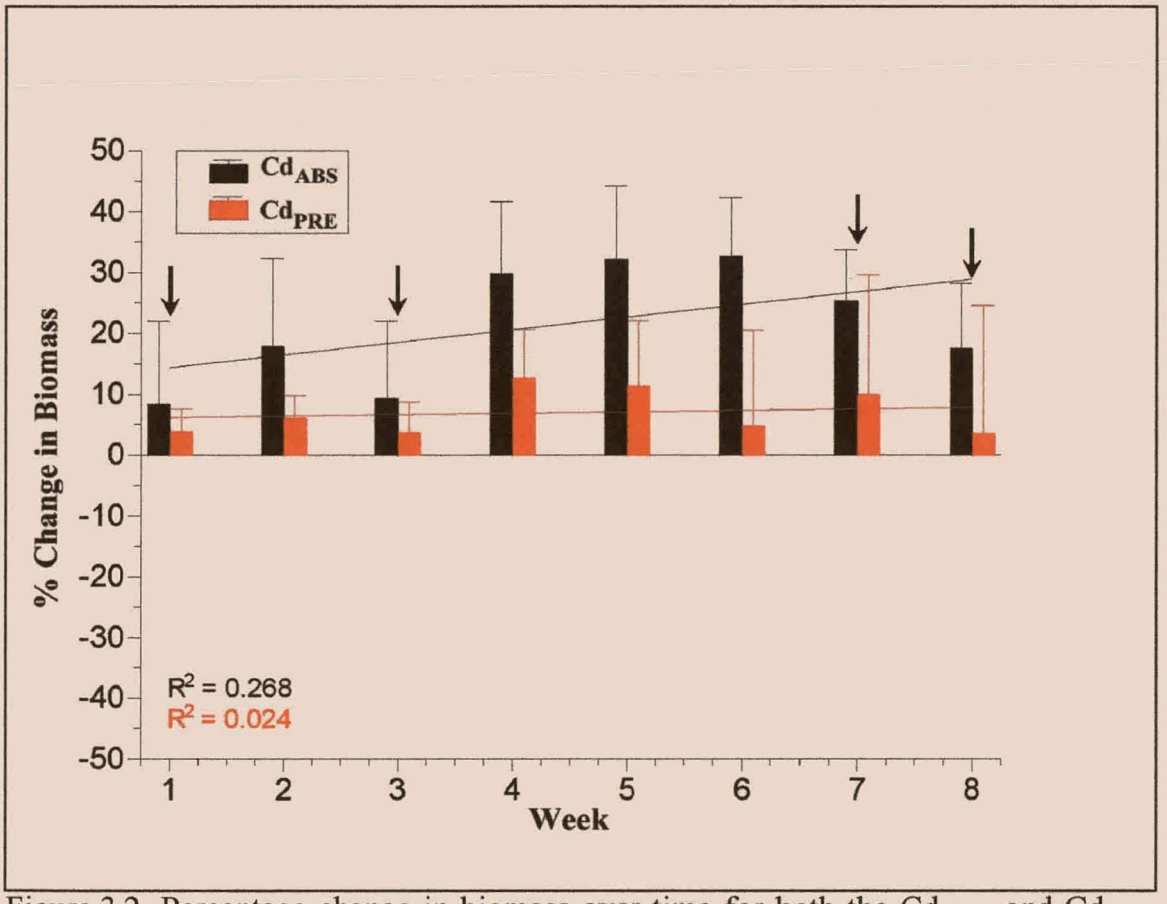

Figure 3.2 Percentage change in biomass over time for both the Cd_{ABS}- and Cd_{PRE}-worms in cattle manure substrate contaminated with 600 $mg.kg^{-1}$ $CdSO_4$. The arrows indicate the weeks where the Cd_{ABS}- and Cd_{PRE}-groups showed no statistically significant difference ($P > 0.001$).

In the substrates contaminated with 1200 $mg.kg^{-1}$ $CdSO_4$, the Cd_{ABS}-worms showed an initial increase in biomass, followed by a decline at the end of the 8-week exposure period. The Cd_{PRE}-worms however, showed a negative change in biomass throughout the 8-week exposure period, accept for the third week where negative change in biomass was observed (Figure 3.3). An ANOVA was carried out ($H = 245.324$ with 15 degrees of freedom, $P < 0.001$). At week 1, no statistically significant difference was observed between the Cd_{ABS} and Cd_{PRE}-groups ($P > 0.001$)

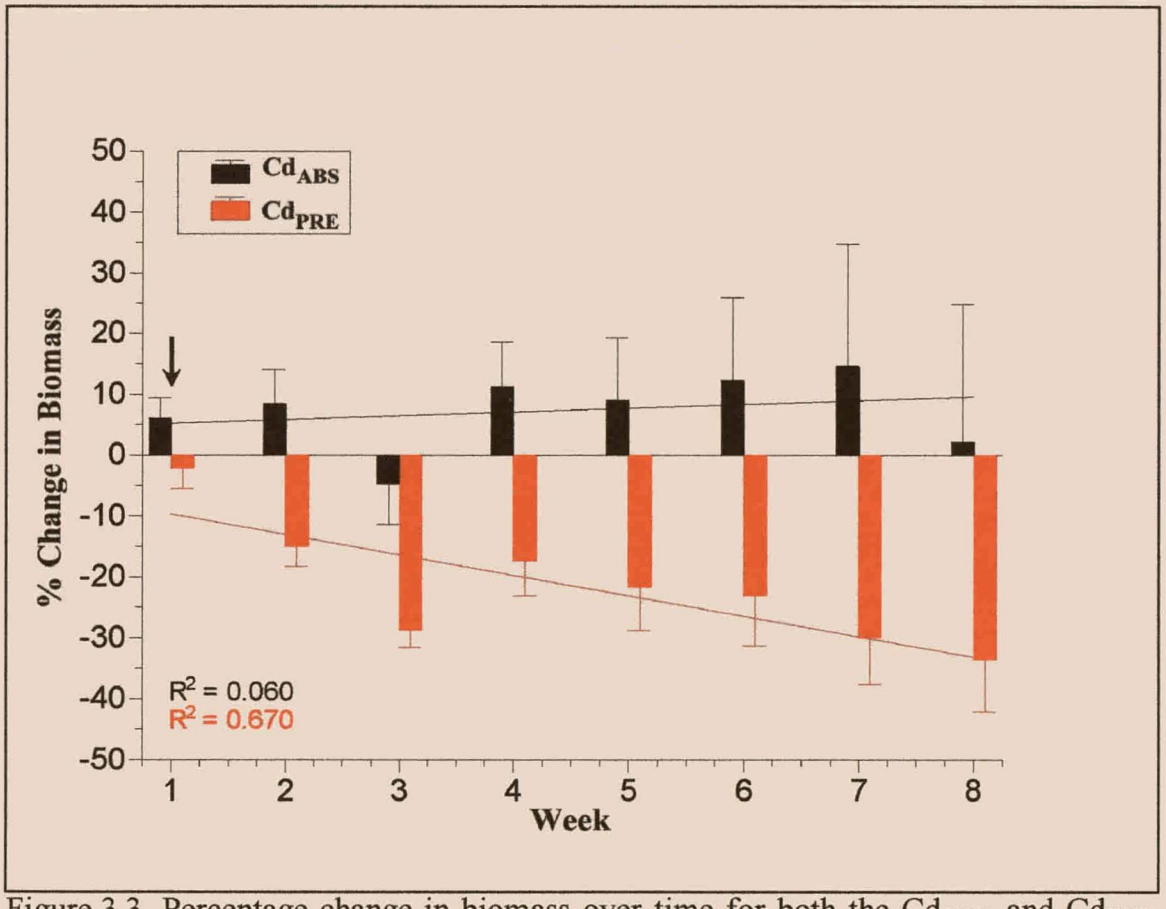

Figure 3.3 Percentage change in biomass over time for both the Cd_{ABS}- and Cd_{PRE}-worms in cattle manure substrate contaminated with 1200 $mg.kg^{-1}$ $CdSO_4$. The arrows indicate the weeks where the Cd_{ABS}- and Cd_{PRE}-groups showed no statistically significant difference ($P > 0.001$).

The total cocoon production of the Cd_{ABS}-groups over the eight-week exposure period showed a progressive decline as the $CdSO_4$ concentration of the substrates increased (Figure 3.4). The Cd_{PRE}-groups however did not show this tendency. In the substrate where no $CdSO_4$ was administered, the total cocoon production was lower than in the substrate contaminated to 600 $mg.kg^{-1}$ $CdSO_4$. The lowest cocoon production for both the Cd_{ABS}- and Cd_{PRE}-groups was observed in the substrate contaminated to 1200 $mg.kg^{-1}$ $CdSO_4$ (Figure 3.4).

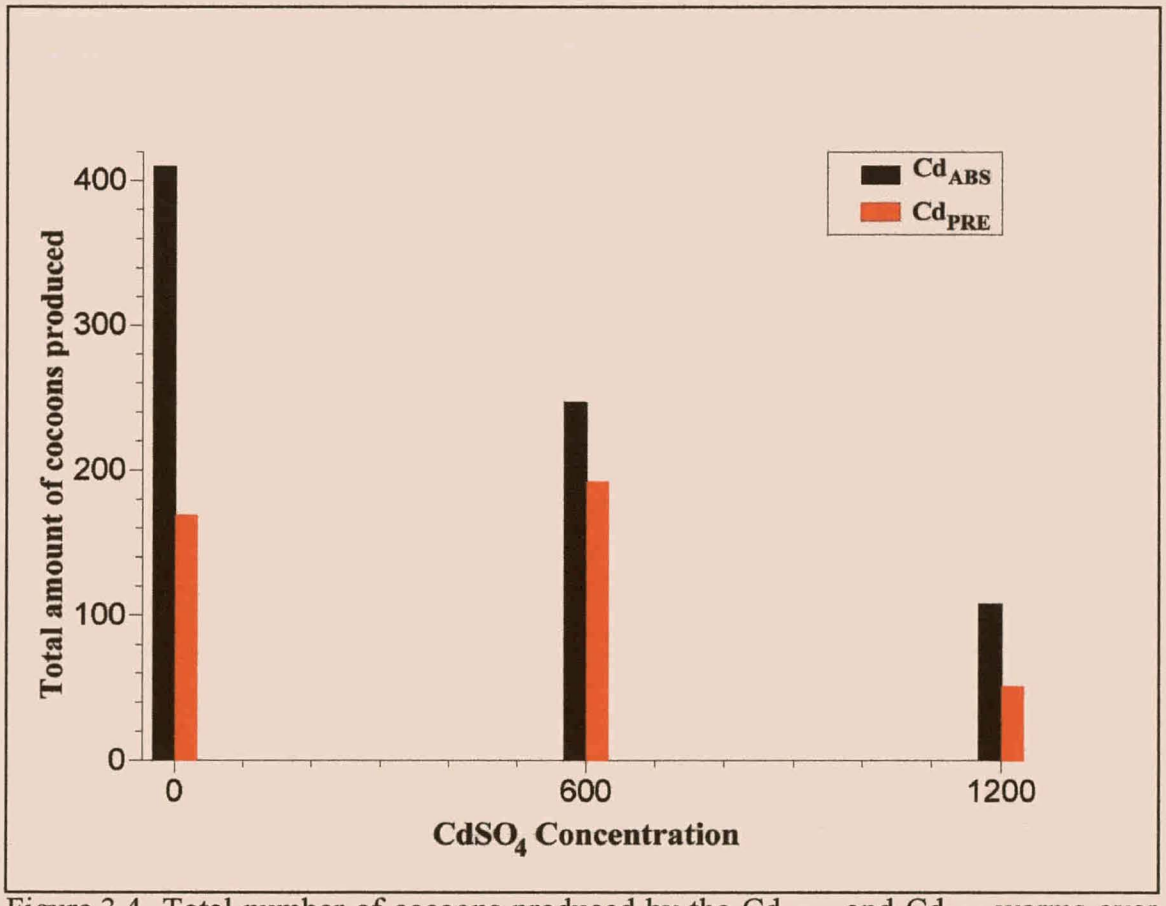

Figure 3.4 Total number of cocoons produced by the Cd_{ABS}- and Cd_{PRE}-worms over the 8-week exposure period to a substrate concentration of 0, 600, and 1200 $mg.kg^{-1}$ $CdSO_4$ respectively

The cocoons produced by the Cd_{PRE}-worms showed a higher percentage of viability than those produced by the Cd_{ABS}-worms at all three concentration levels of $CdSO_4$ (Figure 3.5). For the Cd_{ABS}-groups the values were as follows: at 0 $mg.kg^{-1}$ $CdSO_4$ = 71.9%, at 600 $mg.kg^{-1}$ $CdSO_4$ = 40.7%, and at 1200 $mg.kg^{-1}$ $CdSO_4$ = 35.1%. For the Cd_{PRE}-groups it was as follows: at 0 $mg.kg^{-1}$ $CdSO_4$ = 85.6%, at 600 $mg.kg^{-1}$ $CdSO_4$ = 64.2%, and at 1200 $mg.kg^{-1}$ $CdSO_4$ = 46.5%.

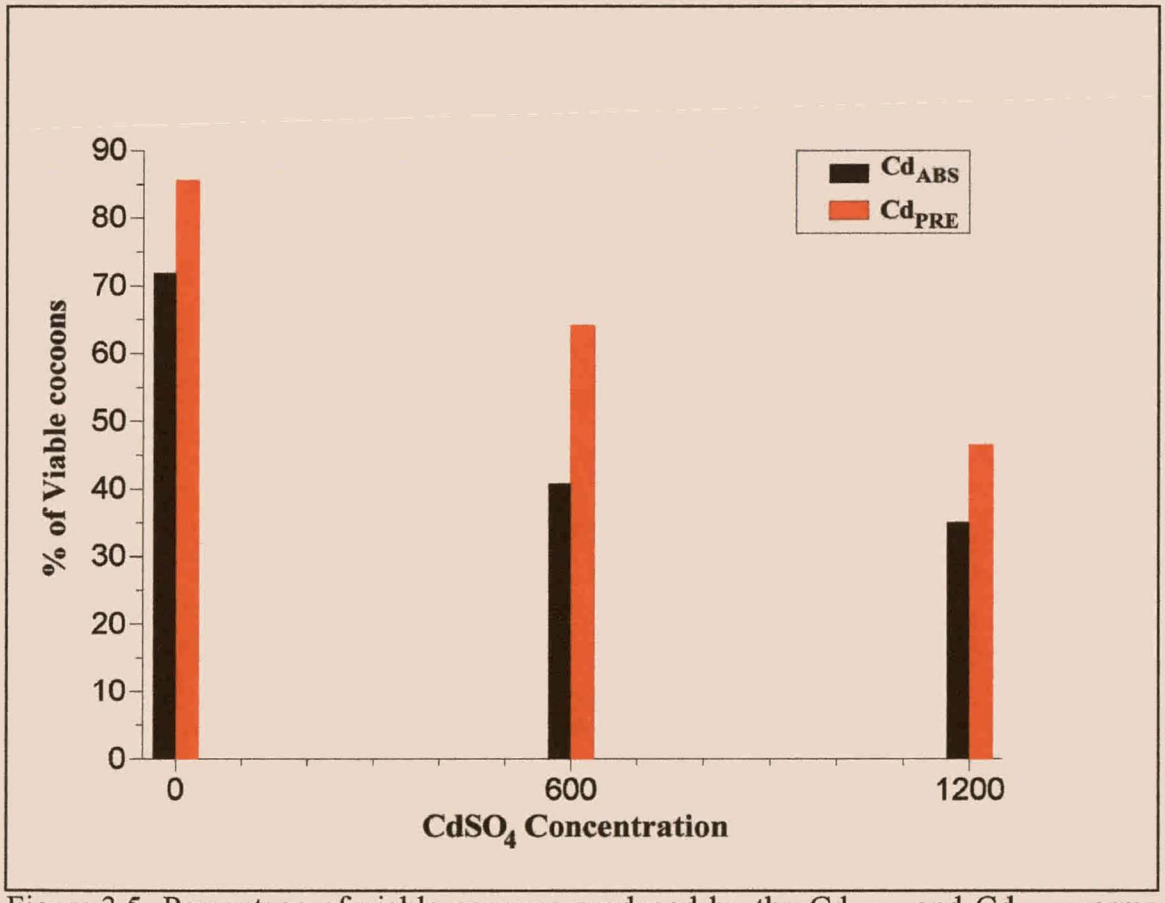

Figure 3.5 Percentage of viable cocoons produced by the Cd_{ABS}- and Cd_{PRE}-worms over the 8-week exposure period to a substrate concentration of 0, 600, and 1200 $mg.kg^{-1}$ $CdSO_4$. Viability was determined over a 28-day period. Cocoons that had not hatched after 28 days were seen as sterile.

The number of worms hatching per cocoon in both the clean uncontaminated and 600 $mg.kg^{-1}$ contaminated substrates was slightly higher for the cocoons of the Cd_{PRE}-worms than for the Cd_{ABS}-worms. In the substrates contaminated to 1200 $mg.kg^{-1}$ $CdSO_4$, however, the amounts of worms hatching per cocoon were slightly higher for the Cd_{ABS}-worms (Figure 3.6). However, statistical analyses of there results using an ANOVA ($H = 24.404$ with 5 degrees of freedom, $P<0.001$), showed that there were no statistically significant differences ($P > 0.001$) between the number of hatchlings produced by the Cd_{ABS}- and Cd_{PRE}-groups at the different concentration levels.

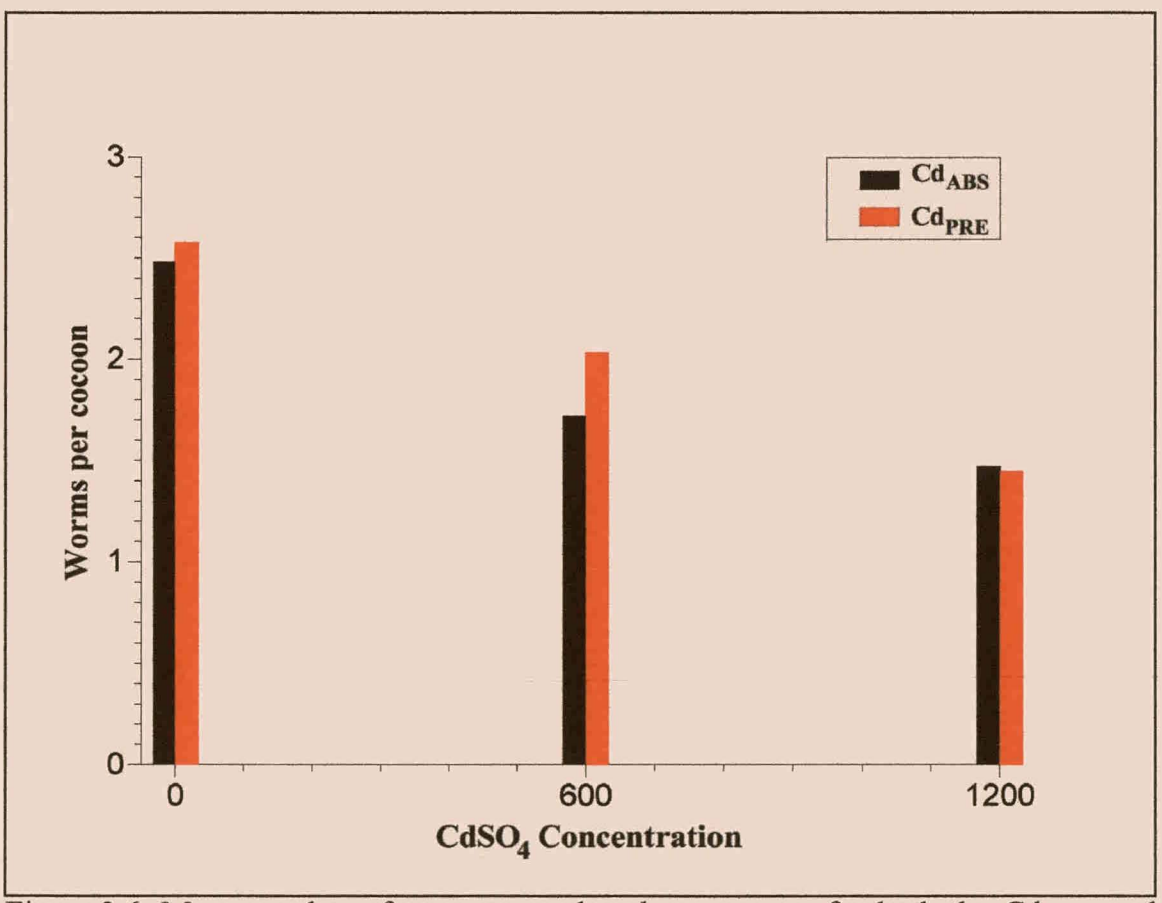

Figure 3.6 Mean number of cocoons produced per cocoon for both the Cd_{ABS}- and Cd_{PRE}-worms exposed to a substrate concentration of 0, 600, and 1200 $mg.kg^{-1}$ $CdSO_4$ respectively.

The mean body concentration (dry weight) of Cd for worms from the Cd_{ABS}- and Cd_{PRE}-groups exposed in the different contaminated substrates was as follows: In the substrates with no $CdSO_4$ added, the [Cd]Cd_{ABS} was 27,62 ± 8.49 $mg.kg^{-1}$ (n=6), and the [Cd]Cd_{PRE} = 211.63 ± 93.28 $mg.kg^{-1}$ (n=6). In the substrate where the worms were exposed to 600 $mg.kg^{-1}$ $CdSO_4$, the [Cd] Cd_{ABS} was 851.44 ± 123.47 $mg.kg^{-1}$ (n=6), and the [Cd] Cd_{PRE} = 775.31 ± 227.02 $mg.kg^{-1}$ (n=6). In the substrate where the worms were exposed to 1200 $mg.kg^{-1}$ $CdSO_4$, the [Cd] Cd_{ABS} was 1170.96 ± 250.13 $mg.kg^{-1}$ (n=6), and the [Cd] Cd_{PRE} = 1343.98 ± 324.24 $mg.kg^{-1}$ (n=6). This is represented in Figure 3.7. Only in the substrate where no $CdSO_4$ was added, a statistically significant difference was found in the mean body concentration of Cd between the Cd_{ABS}- and Cd_{PRE}- worms (ANOVA, H = 30.291 with 5 degrees of freedom, $P < 0.001$)

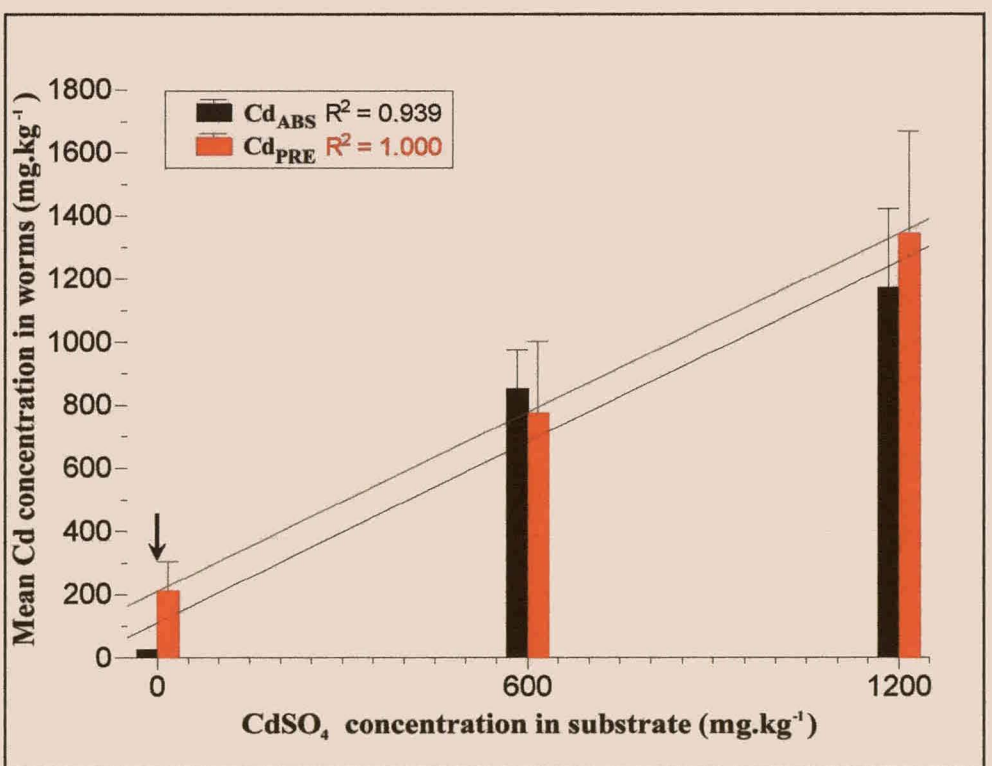

Figure 3.7 Mean body concentration of Cd ($mg.kg^{-1}$) for Cd_{ABS}- and Cd_{PRE}-worms at different concentrations of $CdSO_4$ in the substrate. The arrow indicates the concentration of $CdSO_4$ in the substrate at which the Cd_{ABS}- and Cd_{PRE}-groups showed a statistically significant difference ($P < 0.05$).

No correlation seems to exist between the weekly cocoon production and the weekly change in biomass of the different groups (Figure 3.8)

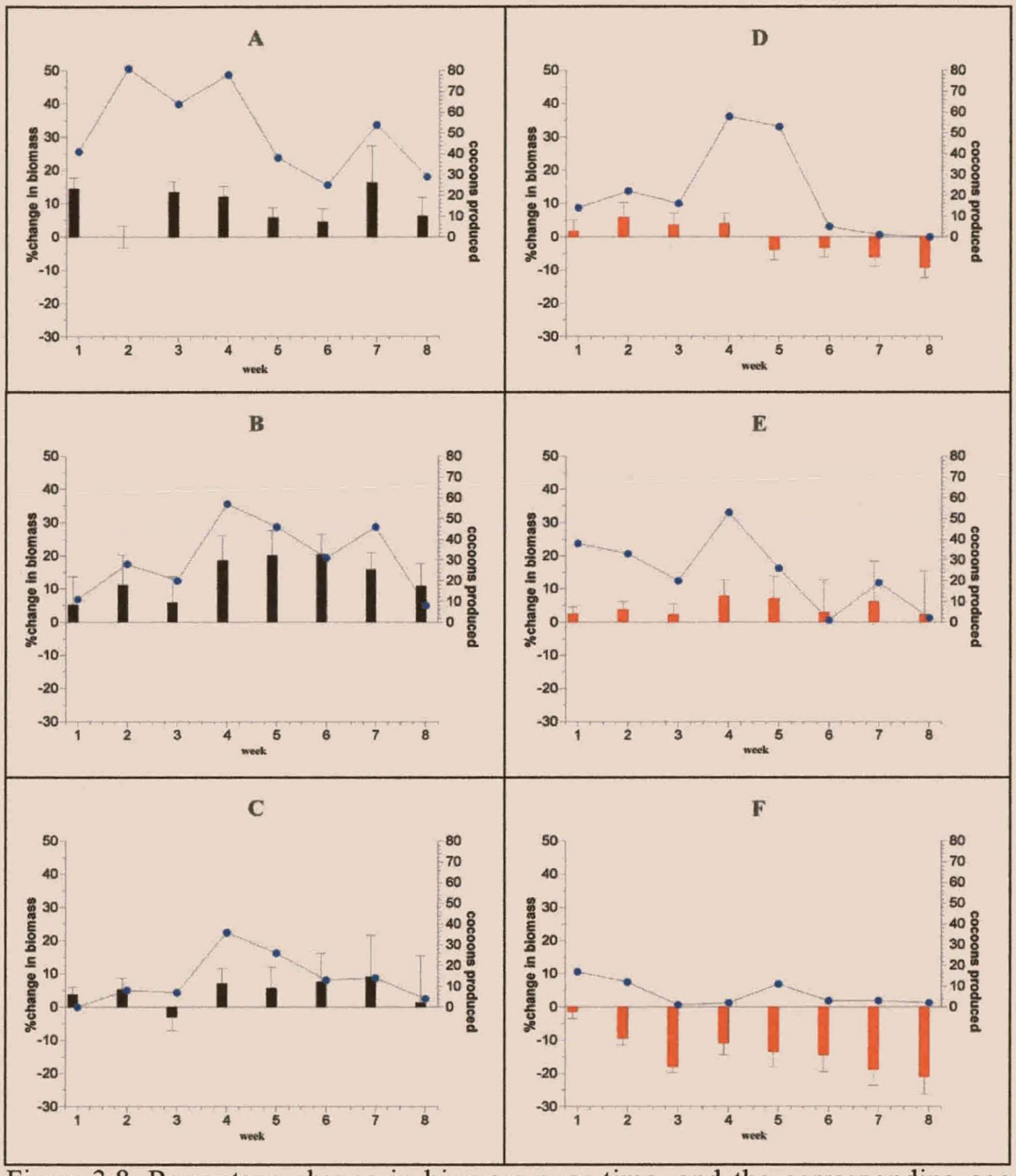

Figure 3.8 Percentage change in biomass over time, and the corresponding cocoon production in: A) Cd_{ABS}-worms in a clean uncontaminated substrate, B) Cd_{ABS}-worms in a substrate contaminated to 600 $mg.kg^{-1}$ $CdSO_4$, C) Cd_{ABS}-worms in a substrate contaminated to 1200 $mg.kg^{-1}$ $CdSO_4$, D) Cd_{PRE}-worms in a clean uncontaminated substrate, E) Cd_{PRE}-worms in a substrate contaminated to 600 $mg.kg^{-1}$ $CdSO_4$, and F) Cd_{PRE}-worms in a substrate contaminated to 1200 $mg.kg^{-1}$ $CdSO_4$. The bar graphs represent the weekly change in biomass, whilst the line graphs represent the weekly cocoon production.

3.4 Discussion

The positive changes in biomass of the Cd_{ABS}-group observed in the substrates where no $CdSO_4$ was administered can be viewed as the normal response of *Eisenia fetida* placed in a new substrate. There was an initial biomass increase in the first week, followed by a drop in the second, whereafter a stable biomass increase in relation to biomass at the beginning of the experiment, was observed. No correlation was found between the change in biomass and cocoon production, although the highest cocoon production per week in this group was observed in the second week (Figure 3.8).

The Cd_{PRE}-group placed in a clean, uncontaminated, substrate showed an initial increase in biomass up to the fourth week. Thereafter a negative increase in biomass was observed (Figure 3.1). A similar effect was found by Reinecke *et al.* (1999) where a similar experiment was conducted in which pre-exposed worms were placed in a clean cattle manure substrate over a five-week period. The authors observed a positive change in biomass for four weeks, whereafter a negative change occurred. In the experiment conducted by Reinecke *et al.* (1999) there was no food supplementation, and the negative response observed by the authors could be ascribed to high levels of stress, because it is expected that heavy metal adaptation will cause a reduction in fitness, which is expressed as non-tolerance characteristics when compared to reference animals (Posthuma & Van Straalen, 1993). Given the development of resistance at a cost, one could argue that the pre-exposed worms need energy to keep the accumulated Cd in the body bound to their respective proteins and organelles (Stürzenbaum, Kille & Morgan, 1988; Brown, 1982). When the quality of food in the substrate falls below a certain critical level the worms are unable to effectively cope with the accumulated Cd. This results in the worms being poisoned

by the accumulated Cd as it is released from its storage compartments within the body. Reinecke *et al.* (1999) however attributed the observed decrease in biomass of the pre-exposed worms after the fourth week in the uncontaminated substrate, to a possible dependency of the animals to Cd. In the experiment conducted in the present study, there was no decrease in food quality of the worms, in fact, the substrate was replaced after the third week and again after the sixth week. Still, after the fourth week, a negative change in biomass was observed, and this negative trend continued up to week eight, when the experiment was terminated. Since a deficiency in food could not account for this negative change in biomass observed in these animals, it may be explained by a developed Cd-dependency in the pre-exposed animals, as proposed by Reinecke *et al.* (1999).

Both the Cd_{ABS}- and Cd_{PRE}-groups showed a positive change in biomass in the substrate contaminated to 600 $mg.kg^{-1}$ $CdSO_4$ (Figure 3.2). This indicates that the concentration level of Cd in the substrate falls within the range of natural tolerance for both groups.

At the highest exposure concentration (1200 $mg.kg^{-1}$ $CdSO_4$), the Cd_{ABS}-group maintained their mean biomass, while the Cd_{PRE}-group displayed a negative change in biomass throughout the duration of the experiment. This is in contrast to what was observed by Reinecke *et al.* (1999), where the pre-exposed worms maintained their biomass throughout the five week experiment, but the control worms showed the negative trend throughout the duration of that experiment. It could be argue that the Cd_{PRE}-group had already accumulated Cd to just under the critical value in the Cd-contaminated culture from which they originated (see section 2.3.1), and that the increase in absorption of Cd from the experimental substrate increased the body Cd levels in these worms to toxic levels. If, however, one considers the mean

concentration of Cd accumulated within the bodies (Figure 3.7) of the Cd_{ABS}- and Cd_{PRE}-worms exposed to 1200 $mg.kg^{-1}$ $CdSO_4$, no statistically significant difference ($P > 0.05$) was found. These concentrations however only represent the amount of accumulated Cd at the end of the experiment, and do not represent the accumulation pattern throughout the course of experiment. At the end of the eight weeks (Figure 3.3) it was found that the mean percentage of growth in the Cd_{ABS}-group was close to 0. This could be an indication that the level of Cd accumulated within the bodies of the two groups was indeed above the critical level at the end of the exposure period. For the Cd_{PRE}-group, however, it may only have taken one week in the heavily contaminated substrate to show the negative effects, while the Cd_{ABS}-group accumulated the Cd throughout the experiment, which only at the end of the experiment exceeded the critical value. To determine whether this is indeed the case, a similar experiment could be conducted for a longer period of time. Alternatively, the Cd accumulation-rate for Cd_{ABS}-worms at a substrate concentration of 1200 $mg.kg^{-1}$ $CdSO_4$ could be determined, to determine how long it takes for Cd to exceed the critical level within these worms.

Cocoon production in all three experimental substrate concentrations was higher for the Cd_{ABS}-groups than for the Cd_{PRE}-groups (Figure 3.4), with the Cd_{ABS}-groups showing a linear decrease in the production of cocoons as the concentration of $CdSO_4$ in the substrate increased. The cocoon production of the Cd_{PRE}-groups however, did not show this trend. The highest number of cocoons was produced in the moderately contaminated substrate (600 $mg.kg^{-1}$ $CdSO_4$), followed by the group that received no exposure, and the lowest number of cocoons were produced by the group in the heavily contaminated substrate (1200 $mg.kg^{-1}$ $CdSO_4$). This lends further evidence to possible Cd-dependency being present in the Cd_{PRE}-group: Not only did

the Cd_{PRE}-group show a negative change in biomass after the fourth week in a clean substrate, but they also produced fewer cocoons in the uncontaminated substrate than in the substrate contaminated to 600 $mg.kg^{-1}$ $CdSO_4$. The low cocoon production in the substrate with the highest concentration of $CdSO_4$ could be a reflection of the concentration of Cd accumulated within the body exceeding the critical level. These findings differ from the findings of Reinecke *et al.* (1999), where both the pre-exposed, and control groups showed a linear decrease in cocoon production as the concentration of $CdSO_4$ in the substrate increased.

However, if one considers the percentage of viable cocoons at the different substrate concentrations, it was found that in all three cases the Cd_{PRE}-groups displayed a higher percentage of viable cocoons (Figure 3.5). This again differs from the findings of Reinecke *et al.* (1999) where the percentage of viable cocoons was found to be higher in the pre-exposed group in the uncontaminated substrate, but lower than that of the control group in the contaminated substrates. The authors ascribed the lower percentage of viable cocoons in the pre-exposed groups in the contaminated substrates to a probable Cd-related sperm damage. The results obtained from the current experiment however seems to contradict this. It may be that Cd does indeed cause sperm damage that results in the linear decrease in viable cocoons as the concentration of $CdSO_4$ in the substrate increases. However, this does not explain why the Cd_{PRE}-groups displayed higher cocoon viabilities in all three substrates. Even in the substrate where no $CdSO_4$ was introduced, and the Cd_{PRE}-worms showed signs of dependency for Cd, the percentage of viable cocoons was more than 20% higher than that of the corresponding Cd_{ABS}-group. Throughout, the percentages of viable cocoons were in all cases higher than those presented by Reinecke *et al.* (1999). In the cocoon viability experiment conducted during this study, the highest percentage of

viable cocoons was 85.6% (for the Cd_{PRE}-group at 0 $mg.kg^{-1}$ $CdSO_4$) and the lowest 35.1% (For the Cd_{ABS}-group in 1200 $mg.kg^{-1}$ $CdSO_4$), in contrast to the findings presented by Reinecke *et al.* (1999), where the highest percentage of viable cocoons did not exceed 30%. This could be explained by the fact that the cocoons were hatched in multicell containers filled with cattle manure substrate, where as the cocoons in the study conducted by Reinecke *et al.* (1999) were hatched in multicell containers filled with distilled water. These results indicate that cattle manure substrate presents a better medium for hatching cocoons of *E. fetida* than distilled water. This may be because there is more aeration of the cocoons than is the case in water. Another factor could be that the distilled water enters the cocoons, thus disrupting the development process inside the cocoon. The results on cocoon viability give a strong indication of a possible resistance mechanism existing in the Cd_{PRE}-worms and should be further investigated.

As for the number of worms produced per cocoon (Figure 3.6), there was a slightly higher number of worms hatching per cocoon for the Cd_{PRE}-groups in the substrates with a $CdSO_4$ concentration of 0- and 600 $mg.kg^{-1}$ $CdSO_4$. At no concentration level, however, were the differences in the number of worms produced per cocoon between the Cd_{ABS}- and Cd_{PRE}-groups statistically significant ($P > 0.05$). In both the Cd_{ABS}- and Cd_{PRE}-groups an almost linear decrease in the mean amount of worms hatching per cocoon was found, as the percentage of $CdSO_4$ in the substrates increased. This may once more be an indication of sperm damage caused by the Cd, being reflected by the decrease in the mean amount of worms hatching from each viable cocoon.

The changes in biomass, and the numbers of cocoons produced, give a strong indication that the Cd_{PRE}-worms have developed a Cd dependency. From the cocoon

viability experiment it also seems as if the cocoons of the Cd_{PRE}-groups are more viable not only in the $CdSO_4$ contaminated substrates, but also in the uncontaminated substrate. These results seem to indicate that there is indeed not only a dependency on Cd in the Cd_{PRE}-worms, but also an indication of a possible resistance to Cd. Whether this dependency and resistance are the results of acclimation, or a genetic tolerance, is not possible to determine from these experiments. It is suggested that further studies should be performed, possibly by conducting similar experiments on the F1, F2 or other generations of the Cd_{PRE}-worms raised in a clean, uncontaminated substrate, comparing them to control worms.

Chapter 4

Effects of cadmium on the nephridia and water homeostasis

4.1. Introduction

It is known that cadmium accumulates in the kidneys of mammals (Elinder, 1977; Roels *et al.*, 1982; Elinder & Järup, 1996; Leffler & Nyholm, 1996; Lind *et al.*, 1998; and Reeves & Vanderpool, 1998) and some marine invertebrates, especially the bivalves (Carmichael, Squibb, Engel & Fowler, 1979; Fowler & Megginson, 1986; and Fowler & Gould, 1988).

In a study conducted by Swiergosz, Zakrzewska, Sawicka-Kapusta, Bacia & Janowska (1998), on the accumulation of cadmium and its effects on bank vole tissues, it was found that the kidneys of cadmium-treated groups exhibited signs of degeneration of the proximal tubules. Although other tissues such as the liver were also affected, the authors attributed the high incidence of mortality in cadmium-treated voles to renal dysfunction. Tang, Sadovic & Shaikh (1998), and Mitsomori, Shibutani, Sato, Onodera, Nakagawa, Hayashi & Ando (1998), reported degradation of the tubular epithelial cells of the proximal tubules of the kidneys of rats and mice, after exposure to Cd. Ishido, Homma-Takeda, Tohyama & Suzuki (1998), conducted a study on the effect of cadmium metallothionein on cultured proximal tubular epithelial cells of rats. They found that there was an induction of Cd-binding proteins and an increase in DNA fragmentation.

Carmichael *et al.* (1979) found that the kidney is a target organ for the bioaccumulation of trace metals in the clam *Mercenaria mercenaria.* In the scallop *Argopecten irradians* exposure to $CdCl_2$ resulted in morphological damage that

consisted of cytoplasmic degeneration in a few focal areas of the exposed kidneys (Carmichael & Fowler, 1981). Fowler and Megginson (1986) found that the kidneys of the scallop, *Placopecten magellanicus* produce a binding protein that forms the major intracellular depot for Cd, Zn and Cu under environmental exposure conditions. In 1988 Fowler and Gould conducted a study on the ultrastructural and biochemical patterns in kidney tubule cells of the *P. maggelanicus* after exposure to Cd. They found that at a low concentration (20 $\mu g.l^{-1}$), no ultrastructural pathology was observed, even though the Cd altered the normal intracellular binding patterns of a number of essential metals in both kidney concretions and systolic metal-binding fractions. The authors concluded that although the Cd-accumulation resulted in specific changes in the metal composition within the cellular compartments, the observed changes did not appear to fall outside the range of cellular homeostasis.

In experiments conducted on the earthworm *E. fetida* to determine the LC_{50} of these animals towards $CdSO_4$ in our laboratory, it was observed that some of the exposed worms had a swollen, translucent appearance (Reinecke *et al.*, 1999). This phenomenon occurred at concentrations of 2000 $mg.kg^{-1}$ $CdSO_4$, and higher. This prompted further investigation into the effect of $CdSO_4$ on the nephridia, and the implications thereof on the water homeostasis of the worms. The present study was therefore conducted to answer some of the questions related to nephrotoxicity of $CdSO_4$ in the earthworm *E. fetida*.

The questions addressed in this chapter were: Firstly, whether or not exposure to $CdSO_4$ would affect the nephridia of exposed worms and cause nephridial damage. Although the nephridial morphology of some spicies of the family Lumbricidae, e.g. *L. terrestris* is available in the literature (Jamieson, 1981; Stephenson, 1930), no references to the morphology of the nephridia of *E. fetida* could be obtained.

Therefore the normal nephridial morphology of unexposed worms had to be determined first, to pinpoint where nephridial damage, if any, occurred in the exposed groups. Secondly, whether exposure to cadmium caused damage to the tissues of these organs, thus disrupting their osmoregulatory function, resulting in a disruption in the osmolality of the coelomic fluid. Withers (1992) gives some information on the osmolality of the extracellular fluid for some freshwater Annelids: *Lumbricus* 299 mOsm, *Pheretima* 152 mOsm, and *Hirudo* 201 mOsm. However, no information on the osmolality of the coelomic fluid for terrestrial annelids, especially *E. Fetida*, could be obtained from the literature (Mill, 1978; Withers, 1992). Therefore the mean osmolality of unexposed worms were determined.

Since no information on the mean number of coelomocites present in the coelomic fluid could be obtained from the literature, it was decided to first determine the mean cell percentage of unexposed worms. The cell percentages of exposed worms of both the Cd_{ABS}- and Cd_{PRE}-groups were then measured to determine whether the cell percentage of the coelomic fluid was affected by exposure to $CdSO_4$. Since it is known that a change in the hematocrit (cell percentage in the blood) in humans and other vertebrates can be used as indicator of physiological changes (Birchard, Black, Schuett & Black, 1984), the cell percentage of the coelomic fluid in earthworms could possibly be used in the same way.

To study these questions, an unexposed control group (Cd_K-), an exposed group in which the worms had no prior history of exposure to any of the non-essential heavy metals (Cd_{ABS}-), and an exposed group of worms that had a history of pre-exposure to cadmium (Cd_{PRE}-), were compared in order to determine whether Cd also causes a nephrotoxic effect in the earthworms. These results were then used to

determine whether a possible increased tolerance to cadmium had developed in worms of the Cd_{PRE}-group in contrast with the Cd_{ABS}-group.

4.2. Materials and methods

4.2.1 Exposure

Three groups of clitellate worms of the species *E. fetida*, originating from cultures maintained in the laboratory, were used (see section 2.3.1). The first group consisted of 10 worms from a control culture, in which no heavy metals had been added to the substrate. This group was used as the unexposed control group (Cd_K-). The second group consisted of 30 worms, also originating from a control culture. This group was exposed to $CdSO_4$, thus serving as an exposed control (Cd_{ABS}-). The third group also consisted of 30 worms. These worms however originated from a population that had been in a substrate, which was contaminated with cadmium for four years. This group served an exposed experimental group (Cd_{PRE}-).

All three groups were placed in separate artificial soil substrates (see section 2.3.2) for the duration of 14 days. No $CdSO_4$ was administered to the artificial soil substrate in which the Cd_K-group was placed, while the substrates of the Cd_{ABS}- and Cd_{PRE}- groups was contaminated with $CdSO_4$ to a concentration between 2000 and 3000 $mg.kg^{-1}$. This concentration range was chosen, because it was at these concentrations that the bloated effect was observed by Reinecke *et al.* (1999).

Cd_{ABS}-worms =Worms originating from a culture where they received no exposure to any heavy metals over a period of four years

Cd_{PRE}-worms =Worms originating from a culture where Cd had been administered over a period of four years

Cd_K-worms =Control group, consisting of worms originating from the same culture as the Cd_{ABS}-worms, but that received no exposure during the course of the experiment.

4.2.2 Sampling and dissection

After the exposure period, the worms from each group were removed from the substrate and immobilised by placing them on ice. Samples of the coelomic fluid were taken by means of sharpened capillary tubes by piercing the body wall. The coelomic fluid would then flow from the coelomic cavity as a result of the coelomic pressure, and into the capillary tubules by means of capillary pressure. After the coelomic fluid samples were taken, samples of the body wall, with the nephridia attached, were dissected out and fixated in Bouin's fluid for histological evaluation (Preece, 1972).

4.2.3 Analyses of coelomic fluid

Directly after the coelomic fluid samples were taken, they were centrifuged by means of a micro-haematocrit centrifuge (Hawksley Micro-Haematocrit Centrifuge, Gelman Instruments) for ten minutes. After centrifugation the percentage of cells in each sample was determined. The cellular component was measured under a dissection microscope by means of a 1 mm grid slide (SFIFT CAT.#M_A663 No.10) calibrated to one hundred 10 µm units. The total volume of the coelomic sample, with inclusion of the cellular component, was measured with a digital calliper (TEAS DIGIT CALL SM). These values were used to determine the cell percentage of the coelomic fluid. After this was done, the osmolality of the supernatant was measured with a vapour pressure osmometer (VAPRO 5520).

4.2.4 Histological preparation of tissue samples

The specimens fixed in Bouin's fluid were dehydrated by a series of increasing ethanol baths, up to 100% ethanol. Final dehydration was done by taking the specimens from 100% ethanol to a 1:1 ethanol:chloroform bath, and then to 100%

chloroform. After the dehydration process was completed, the specimens were imbedded, first into a solution of 1:1 chloroform:paramat, and then in 100% paramat wax under vacuum for 30 minutes. After final imbedding into Paramat wax was completed, a series of sections of 7µm thickness were made. These sections were stained by means on the Mallory-Heidenhain aniline blue method (Preece, 1972), (Appendix 1).

4.2.5 Nephridial reconstruction

A series of sections from Cd_K-worms were used to reconstruct the anatomy of a normal (healthy) nephridium diagrammatically. This was done by projecting and tracing a series of 120 images of consecutive sections onto paper. Measurements were then made on the images and transferred to graph paper, where a schematic reconstruction was made. This reconstruction was done to pinpoint where nephridial damage, if any, had occurred in the nephridia of the exposed groups when examining the histological sections.

4.3 Results

4.3.1 Anatomy of the nephridia

A schematic reconstruction of a normal (unexposed) nephridium is given in Figure 4.1. The nephridia consist of a nephrostome (NS), followed by a single convoluted tubule leading to a first loop (FL). From there convoluted tubuli lead to a much larger, second loop (SL). From the second loop, a single tubule runs parallel to the convoluted tubuli in the direction of the nephrostome, where it opens into a wider ampulla (AMP) which in turn narrows down into a prevasicular tubule (Pre.V). This prevasicular tubule leads in the direction of the second loop, and gives rise to a large urinary vasiculus (UV) where the urine is stored temporarily before it leaves the nephridium via the postvasicular tubule (Post.V), which opens on the outside the body via the nephridiopore (NP).

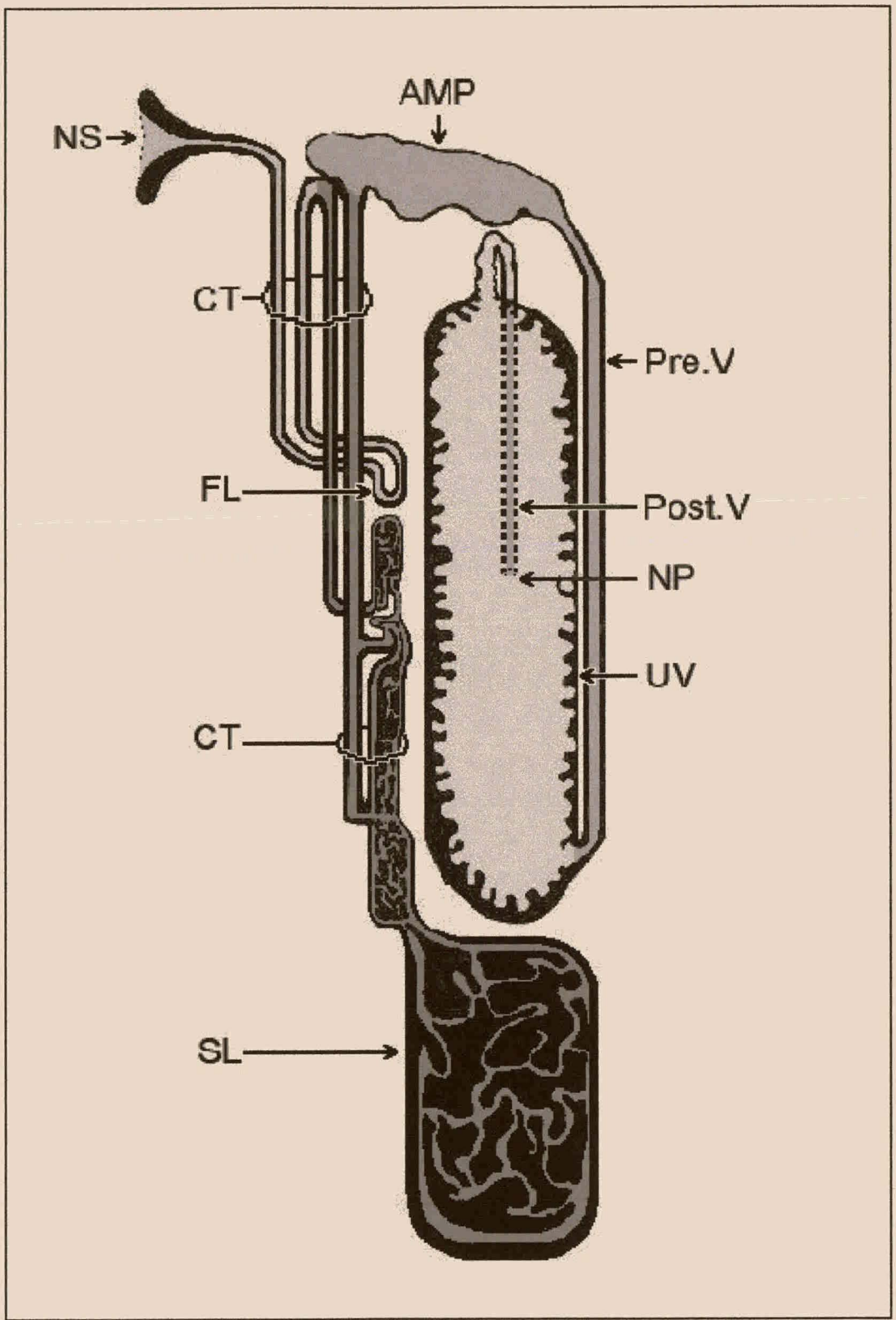

Figure 4.1 Schematic reconstruction of the nephridium of *Eisenia fetida*: (NS) Nephrostome, (CT) Convoluted Tubuli, (FL) First Loop, (SL) Second Loop, (AMP) Ampulla, (Pre.V) Prevasicular Tubule, (UV) Urinary Vasiculus, (Post.V) Postvasicular Tubule, (NP) Nephridiopore.

4.3.2 Nephridial damage

After the 14-day exposure period, some of the exposed worms of both the Cd_{ABS}- and Cd_{PRE}- groups had a bloated, translucent appearance (Appendix 2). If not handled carefully, the body wall of these worms would release a lot of fluid, where after the worms appeared normal. These worms will be referred to as bloated- (^{B}Cd-) worms. Other worms of the exposed groups had a normal appearance, and will be referred to as normal- (^{N}Cd-) worms.

The nephridia of ^{B}Cd-worms of both the Cd_{ABS}- and Cd_{PRE}-groups were much smaller in appearance than the nephridia of the ^{N}Cd-worms of both groups when viewed with the dissection microscope. Histologically, it was found that the second loop of each nephridium from the ^{B}Cd-worms had degenerated. In some instances the degradation of the second loop was so severe, that only a few traces of the tissue surrounding the tubules of the second loop remained, and the tubules themselves had disappeared. The decrease in size of the whole nephridium, observed under the dissection microscope, could therefore be the result of the degeneration of the second loop in the ^{B}Cd-worms. The ^{N}Cd-worms showed a reduction in the amount of fat-like tissue surrounding the second loop, but not to the extent found in the ^{B}Cd worms where virtually all traces of the second loop had disappeared. Thus in the ^{N}Cd-worms, the whole nephridium still looked normal when viewed under the dissection microscope.

^{B}Cd- = Worms that had a bloated, translucent appearance after the exposure period.
^{N}Cd- = Worms that appeared as normal after the exposure period.

Microscopical examination of the histological sections of the nephridia from ^{B}Cd-worms showed clear cellular damage to the convoluted tubuli. This damage was so severe that the individual tubules had disappeared and only an indistinguishable conglomerate of cells remained (Figure 4.2 B). The cellular damage was not only restricted to the convoluted tubuli, but was also observed in the first- and the second loop. The epithelia of the urinary vasiculi from these worms also showed clear signs of degradation, as did the postvasicular tubules that lead to the nephridiopores. The damage described was found in the ^{B}Cd-worms of both the Cd_{ABS}- and Cd_{PRE}-groups.

Nephridia of the ^{N}Cd-worms of both the Cd_{ABS}- and Cd_{PRE}-groups appeared to have remained intact and functional, when compared to those of the Cd_{K}-group (Figure 4.2 A, C). The lumina of the individual convoluted tubuli could still be seen clearly. The first- (Figure 4.2 C) and the second loop where also still present in these worms, as were the convoluted tubuli. The epithelium of the urinary vasiculus also did not show signs of degradation, as was the case with the ^{B}Cd-worms. The prevasicular tubule, although reduced in size, could also still be distinguished (Figure 4.2 C).

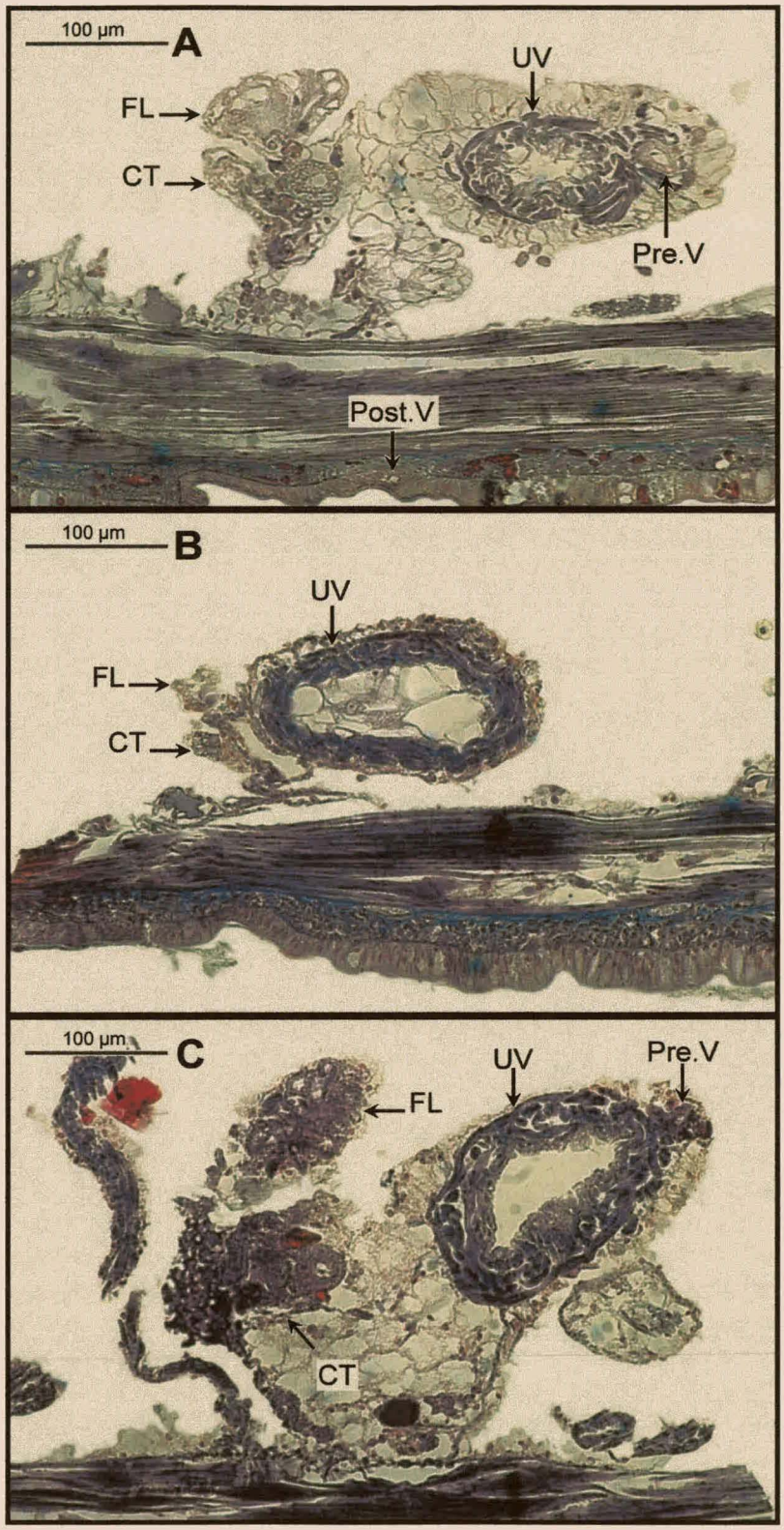

Figure 4.2 Histological section through the region of the first loop of A) an unexposed (control) worm (Cd_K), B) an exposed, bloated worm ($^{B}Cd_{ABS}$-worm) and C) an exposed normal appearing worm ($^{N}Cd_{ABS}$-worm): (CT) Convoluted Tubuli, (FL) First Loop, (UV) Urinary Vasiculus, (Pre.V) Prevasicular Tubule, (PostV) Postvasicular Tubule.

4.3.3 Changes in osmolality and cell percentage of the coelomic fluid

The mean coelomic osmolality of the ^{B}Cd- and ^{N}Cd-worms from different groups is represented in Table 4.1. It was found that in ^{N}Cd-worms of both the Cd_{ABS}- and Cd_{PRE}-groups, there was a slight increase in the osmolality of the coelomic fluid when compared to that of the unexposed Cd_{K}-group. This difference however, was not statistically significant.

Table 4.1. Mean osmolality of the unexposed control group and the experimental groups, after a 14-day exposure to 3000 mg.kg^{-1} $CdSO_4$ in an artificial soil substrate

	Cd_{K}-worms (n=10)	$^{N}Cd_{ABS}$-worms (n=18)	$^{B}Cd_{ABS}$-worms (n=9)	$^{N}Cd_{PRE}$-worms (n=22)	$^{B}Cd_{PRE}$-worms (n=6)
Osmolality (mmol.kg^{-1})	163.40 ± 7.01 a,d	177.33 ± 10.80 b,e	137.22 ± 24.76 a,b,c	180.09 ± 17.39 c,f	132.67 ± 12.31 d,e,f

Cd_{ABS}-worms = Worms originating from a culture where they received no exposure to any heavy metals over a period of more than 3 years

Cd_{PRE}-worms = Worms originating from a culture where Cd had been administered over a period of more than 3 years

Cd_{K}-worms = Control group, consisting of worms originating from the same culture as the Cd_{ABS}-worms, but that received no exposure during the course of the experiment.

^{B}Cd- = Exposed worms that appear bloated

^{N}Cd- = Normal appearing exposed worms

Similar lower case lettering indicates the groups that showed a statistically significant difference ($P < 0.05$) in their osmolality.

There was a statistically significant difference ($P < 0.05$) in the mean coelomic osmolality between the ^{N}Cd- and ^{B}Cd-worms of both the Cd_{ABS}- and Cd_{PRE}-groups. The mean osmolality of the ^{B}Cd-worms of both Cd_{ABS}- and Cd_{PRE}-groups was also statistically significantly lower than that found in the Cd_K-worms. When the mean osmolalities of the ^{B}Cd- and ^{N}Cd-worms of the Cd_{ABS}- and Cd_{PRE}-groups were compared to one another respectively, there were no statistically significant differences found.

The mean cell percentages of the coelomic fluid from the different groups are represented in Table 4.2 The ^{N}Cd-worms of both the Cd_{ABS}- and Cd_{PRE}-groups showed no statistically significant difference in the mean cell percentage of the coelomic fluid when compared to the Cd_K-group ($P > 0.05$). The ^{B}Cd-worms of the Cd_{ABS}-group did however show a statistically significant decrease in the mean cell percentage of the coelomic fluid when compared to the Cd_K-group ($P < 0.05$). The mean cell percentage of the ^{B}Cd-worms from the Cd_{PRE}-group did not differ statistically from that found in the Cd_K-group ($P > 0.05$). When the mean cell percentages of the ^{N}Cd-worms of the Cd_{ABS}- and Cd_{PRE}-groups were compared to one another, there was also no statistically significant difference ($P > 0.05$). A statistically significant difference was however found between the mean cell percentage from the ^{B}Cd-worms of the Cd_{ABS}- and Cd_{PRE}-groups ($P < 0.05$).

Table 4.2 Mean cell percentage of the unexposed control group and the experimental groups, after a 14-day exposure to 3000 $mg.kg^{-1}$ $CdSO_4$ in an artificial soil substrate

	Cd_K-worms (n=10)	$^{N}Cd_{ABS}$-worms (n=18)	$^{B}Cd_{ABS}$-worms (n=9)	$^{N}Cd_{PRE}$-worms (n=22)	$^{B}Cd_{PRE}$-worms (n=6)
Cell-percentage	9.40 ± 2.40 a	8.49 ± 2.36 b	5.16 ± 3.12 a,b,c,d	10.60 ± 4.45 c	9.06 ± 4.65 d

Cd_{ABS}-worms = Worms originating from a culture where they received no exposure to any heavy metals over a period of more than 3 years

Cd_{PRE}-worms = Worms originating from a culture where Cd had been administered over a period of more than 3 years

Cd_K-worms = Control group, consisting of worms originating from the same culture as the Cd_{ABS}-worms, but that received no exposure during the course of the experiment.

^{B}Cd- = Exposed worms that appear bloated

^{N}Cd- = Normal appearing exposed worms

Similar lower case lettering indicates the groups that showed a statistically significant difference ($P < 0.05$) in the cell percentage of their coelomic fluid

4. 4. Discussion

Each nephridium of an earthworm can be compared to a single nephridial unit (nephron) of the mammalian kidney (Withers, 1992). The nephrostome of the earthworm is fairly analogous in function to the glomerulus of the mammalian nephron. The first- and second loops can be compared to the proximal- and distal tubules of the mammalian kidney, both of which are found in the cortex of the kidney.

The tubular damage that was observed in the $^{B}Cd_{ABS}$- and $^{B}Cd_{PRE}$-worms correlates with the damage observed in the proximal tubules of the mammalian kidneys after Cd exposure (Swiergosz *et al.*, 1998; Tang *et al.*, 1998; Mitsomori *et al.*, 1998; and Ishido *et al.*, 1998). Since this degeneration of the nephridial tubules was not observed in the $^{N}Cd_{ABS}$- and $^{N}Cd_{PRE}$-worms, there might be some mechanism in these worms that render their nephridia more resistant to the effects of cadmium.

In mammals an increased excretion of proteins in the urine (proteinuria) and also in some cases an increased excretion of calcium (calcuria) (Nogawa, 1984; Jin, Leffler & Nordberg, 1987; and Taylor, Chivers, Price, Arce-Tomas, Milligan, Francini, Alinovi, Cavazzini, Bergmaschi, Vittori, Mutti, Lauwerys, Bernard, Roels, De Broe, Nuyts, Elseviers, Hotter, Ramis, Rosello, Gelpi, Stolte, Eisenberg & Fels, 1997) indicate degeneration of the nephridia. In the case of *E. fetida*, cadmium related nephridial degeneration seems to be indicated by an increase in coelomic volume.

Ishido *et al.* (1998) conducted a study on the effects of Cd-metallothionein on proximal tubular cells of Jcl:Wistar rats, to determine the effect of Cd on the apoptotic processes of these cells. These authors found that Cd did indeed produce biochemical and morphological alterations which are characteristic of apoptosis. They concluded that the administration of $CdCl_2$ to the animal would induce the synthesis of a Cd-binding metallothionein (Cd-MT) in the liver. This Cd-MT complex is then released

from the liver and transported to the kidney. There the Cd-MT complex is absorbed into the proximal tubules and degraded, releasing the ionic cadmium inside the renal tubular cells. The authors suggest that this ionic cadmium released within the tubular cells induce the toxic effects, which include chromatin condensation and DNA fragmentation. It may well be possible that in the earthworm a similar mechanism exists as suggested by Ishido *et al.* (1998). It is well known that Cd does accumulate in the cloragogenous tissue surrounding the alimentary tract (Hopkin 1989). It is from this region that Stürzenbaum *et al.* (1988) identified, cloned and characterised an earthworm metallothionein in *Lumbricus rubellus*. A Cd excretion pathway in the earthworm may involve the incorporation of the metal into a metallothionein in the cloragogenous tissue surrounding the gut. From here the metallothionein could be transported via the coelomic fluid and circulatory system to the nephridia. There it is then separated from the metallothionein and excreted. If this is indeed the case, it might explain the tubular degeneration in the first and second loops of the nemhridium, for it might be possible that these regions of the nephridium are critical in the Cd-detoxification mechanism of earthworms, as is the case in mammals (Ishido *et al.*, 1998).

The ^{N}Cd-worms of both the Cd_{ABS}- and Cd_{PRE}-groups displayed normal nephridial morphology, as well as a normal cell percentage and osmolality of the coelomic fluid after the exposure period. The normal coelomic cell percentage observed in these animals may be an indication that the nephridia maintained their osmoregulatory function. Histologically it was found that all parts of the nephridia of these worms were still intact, and appeared normal. This would suggest that the different regions of the nephridia remained functional, as no disruption in the water homeostasis could be observed in these worms.

The fact that no statistically significant differences were found between the mean osmolality of the ^{N}Cd-worms of both groups, or between the ^{B}Cd-worms of both groups (Table 4.1), indicates that the nephridia of both the ^{N}Cd- and ^{B}Cd-worms display the same degree of disruption in their physiological function. There is thus no indication of a development of resistance in the nephridia of the Cd_{PRE}-group to Cd if one compares osmolality of the coelomic fluids during exposure to Cd, to that of a control group (Cd_{ABS}-) that received the same treatment. The statistically significant decrease in osmolality between the ^{B}Cd- worms of the Cd_{ABS}- and Cd_{PRE} –groups, in relation to the Cd_{K}-group, might be the result of disruption in the water excretion capacity of the nephridia due to the nephridial degeneration observed in these worms. This would also explain the bloated appearance of these worms.

No statistically significant decrease in the cell percentage of the ^{B}Cd-worms of the Cd_{PRE}-group in relation to the Cd_{K}-group was observed, despite the increase in coelomic fluid volume (Table 4.2). This suggests that although the volume of the coelomic fluid had increased in the ^{B}Cd-worms of the Cd_{PRE}-group, the cell percentage in the coelomic fluid had remained constant. However, the ^{B}Cd-worms of the Cd_{ABS}-group did not only show a statistically significant decrease in the mean cell percentage when compared to the ^{N}Cd-worms of the Cd_{ABS}-group, but also when compared to the ^{B}Cd-, and ^{N}Cd-worms of the Cd_{PRE}-group. This could indicate that the coelomocytes of the ^{B}Cd-worms of the Cd_{ABS}-group were more susceptible to the effects of cadmium than the coelomocytes of the ^{B}Cd-worms of the Cd_{PRE}-group. This could be coupled to a detoxification mechanism present in the Cd_{PRE}-group that is absent in the Cd_{ABS}-group. If one takes into account that there might not have been a decrease in the coelomic cell percentage, but that the increase in the volume of the coelomic fluid give rise to a lowered cell percentage, the capability of the ^{B}Cd-worms

of the Cd_{PRE}-group to maintain a normal cell percentage is even more astounding, as it could indicate an increase of the coelomocite numbers.

This study has revealed that nephridial damage similar to that in the mammalian kidney is caused in some specimens of *E. fetida* when exposed to high concentrations of $CdSO_4$. It has also been shown that measurements of the cell percentage of the coelomic fluid may give an indication of a disruption in the osmoregulatory capacity of the worms, especially when viewed in relation to the changes in the osmolality. Furthermore, there is an indication that worms that have historically been exposed to Cd- may possess a mechanism that renders their coelomocytes more resistant to the effects of Cd. This mechanism might involve an increased tolerance of the coelomocytes to the Cd in the coelomic fluid. Alternatively there might be some mechanism that allows the nephridia to extract enough Cd from the coelomic fluid causing a decrease in the coelomic Cd-concentrations to a level low enough for coelomocite survival. To test this hypothesis, future experiments could be conducted to determine the Cd-concentration in the coelomic fluid of Cd_{ABS}- and Cd_{PRE}-worms exposed to cadmium. This could be accomplished by the sprayed microdroplets technique (Morgan & Winters, 1993)

CHAPTER 5

Accumulation of cadmium in the nephridia

5.1 Introduction

Critical organs for cadmium toxicity in animals are the excretory organs. Cadmium has been shown to accumulate in the kidneys of human beings (Elinder, 1977; Roels, Lauwerys *et al.*, 1981; Lauwerys *et al.*, 1992) and other mammals such as goats (Khan, Diffay, Datiri, Forester, Thompson & Mielke, 1995), mice (Lind *et al.*, 1998), rats (Mitsumori *et al.*, 1998) and bank voles (Leffler & Nyholm, 1996; Swiergosz *et al.*, 1998). Cadmium accumulation has also been noted in the excretory organs of invertebrates, such as the clams *Mercenaria mercenaria* (Carmichael *et al.*, 1979) and *Macoma balthica* (McLeese & Ray, 1984) as well as in the scallops *Argopecten irradians* (Carmichael & Fowler, 1981) and *Placopecten magellanicus* (Fowler & Megginson, 1986; Fowler & Gould, 1988).

The accumulation in humans was found to be the highest in the cortex of the kidney (Elinder, 1977; Roels *et al.*, 1981; Lauwerys *et al.*, 1992; and Torra, To-Figueras, Brunet, Rodamilans & Corbella, 1994). This also holds true for the small mammals (Mitsumori *et al.*, 1998). In mammals, the first signs of nephrotoxicity are observed in the epithelial cells of the proximal tubules, which are situated in the cortex region of the kidney. Metallothioneins are thought to play a role in the nephrotoxicity. In the blood, cadmium binds to the metallothioneins that are produced by the liver. When the Cd-metallothionein complex reaches the glomerulus, it is freely filtered through the renal glomeruli and absorbed into the proximal tubular

epithelial cells (Tang *et al.*, 1998). In these cells, lysosomal enzymes rapidly degrade the Cd-metallothionein complex, and the free Cd^{2+}-ions are released within the cells. The Cd^{2+} can now be incorporated in endogenous metallothioneins. Excess Cd^{2+} may however interact with critical cellular organelles, such as the mitochondria, and inhibit Ca^{2+} translocation (Tang *et al.*, 1997). In the small mammals Cd is also bound to a metallothionein, and a similar mechanism of Cd transport and the associated proximal tubular degeneration is suggested (Ishido *et al.*, 1998).

Whether such a mechanism exists in invertebrates is not known. In a study conducted by Carmichael *et al.* (1979) on the clam *Mercenaria mercenaria* to determine the metal uptake and distribution of radio labelled ^{109}Cd, ^{54}Mn and ^{65}Zn, it was found that a small portion of ^{109}Cd was incorporated in nephridial concretions. The authors attributed this to a slow incorporation of Cd into the concretions, since high Cd concentrations in nephridial concretions were observed in other studies. The authors also found a Cd-binding metallothionein, isolated from the nephridia. However, whether this was a transport- or endogenous-metallothionein was not specified by the authors.

In the scallop *Placopecten magellanicus*, exposure to 20 $\mu g.l^{-1}$ Cd caused a 6.5-fold increase in the total nephridial Cd concentration when compared to unexposed controls (Fowler & Gould, 1988). The authors ascribed the lack of an observed toxicity in the exposed animals, to the effective sequestration of Cd within the nephridial concretions and cytosolic metal-binding protein compartments. Exposure caused a 6- fold increase of the Cd/Ca ratio of the concretions, and a 6.6-fold increase in the cytosol Cd fraction (Fowler & Gould, 1988). It was concluded that Cd-exposure did not produce ultrastructural pathology in nephridia of the exposed animals, and that although Cd accumulation in the different compartments was

associated with changes in the normal metal composition, the Cd-induced changes did not fall outside the range of homeostasis.

Studies on the nephridial accumulation in the nephridia of terrestrial invertebrates could not be obtained. However, Reinecke *et al.* (1999) referred to a possible disruption in the water homeostasis of the earthworm *E. fetida* after exposure to high concentrations of $CdSO_4$. In the previous chapter, the nephrotoxic effect of $CdSO_4$ was investigated, for both exposed worms originating from an unexposed stock culture, and worms originating from a stock culture where they were pre-exposed to Cd in the form of $CdSO_4$.

The questions posed in this chapter are firstly, whether Cd would accumulate in the nephridia of exposed earthworms and secondly, if Cd did accumulate in the nephridia, would there be any differences in the Cd-concentrations in the nephridia from pre-exposed (Cd_{PRE}-) and control (Cd_{ABS}-) worms after exposure to high concentrations of $CdSO_4$.

Cd_{ABS}-worms =Worms originating from a culture where they received no exposure to any heavy metals over a period of four years

Cd_{PRE}-worms =Worms originating from a culture where Cd had been administered over a period of four years

5.2 Materials and methods

5.2.1 Background to Particle induced X-ray emission:

There are numerous ways of determining the metal concentration in a biological sample. These include acid digestion and analysis of the specimen by means of an atomic absorption spectrophotometer (Yamamoto, Mori & Suzuki, 1987; Khan *et al.*, 1995; Reeves & Vanderpool, 1998). This method is suited to rather large samples, usually in the order of 1 g. For the determination of the metal content in a very small tissue samples X-ray analyses using a scanning electron microscope (EDAX) is often used (George, Pirie, Coombs, 1980; Carmichael & Fowler, 1981). The method we employed during the present study for analysing tissue samples is perhaps a less well known, but more sensitive technique, known as particle induced X-ray emission (PIXE).

Particle induced X-ray emission has various applications in determining the elemental composition of materials which range from the analyses of water samples (Johansson & Johansson, 1984), soil samples (Protz, Teesdale, Maxwell, Campbell & Duke, 1993), aerosol samples (Malmqvist, 1994), fragile archaeological specimens (Buoso, Fazinic, Haque, Moschini, Volpe & Caravello, 1992), plant tissues (Malmqvist, 1994), and animal tissues (Kieth, Gandolfi, McIntyre, Ashbaugh & Fernando, 1997).

In the present study PIXE was used to determine the accumulation and distribution of cadmium in the nephridia of earthworms after exposure to $CdSO_4$. Since this technique is not widely used, a short overview of proton induced X-ray emission will follow. In the overview emphasis will be placed on what a nuclear microprobe is, the general characteristics of ion beams, proton induced X-ray

emissions, the data acquisition system, how thick targets are analysed and lastly the difference between the PIXE and EDAX techniques.

The nuclear microprobe is an analytical instrument that makes use of different high-energy ions generated by particle accelerators. The accelerator generates particles with energies of MeV (10^6 eV) as compared to keV (10^3 eV) for electrons used in electron microscopy (Tapper & Malmqvist, 1991). The particles used in a nuclear microprobe can range from protons to heavier ions. The particles are accelerated in a particle beam and focused on a target (sample). As the ions interact with the target matter, their velocity decreases, producing useful signals for qualitative or quantitative analyses. Signals that can be produced by the interaction of the particles with a specimen are visible light, characteristic X-rays, Auger and secondary electrons, photons or charged particles from nuclear reactions, and scattered ions (Figure 5.1). Any one of these signals can be used for the characterisation of the specimen (Tapper & Malmqvist, 1991). Two important techniques for determining the elemental content of a sample are the particle induced X-ray emission (PIXE) and Rutherford backscattering spectroscopy (RBS). Of these two PIXE has the highest analytical sensitivity.

Ion beams (protons, deuterons, alpha particles or other ions) can be focused down to a minimum size of ~1 μm in a diameter (Johansson. & Campbell, 1988). This is accomplished by a special lens system. Unlike electron microscopes where the electron beam can be focused by means of an electromagnetic lens, for example a solenoid, the magnetic field required for the focusing of protons is considerably higher. The high current density needed would lead to overheating of the coil. Therefore, nuclear microprobes make use of quadrupole lenses for focussing. A set of at least two quadrupole lenses is required to obtain an image of the object aperture on

the sample. A single point analyses in the region equal to/or larger than 1 μm is thus achievable (Johansson & Campbell, 1988). Alternatively the beam can be scanned over areas of up to 1.5 x 1.5 mm.

When the incident MeV-ions interact with the specimen atoms, electrons are emitted from the inner shells, which when filled by electrons from higher shells release excess energy either as an emitted photon (X-ray emission) or an electron (Auger electron emission) (Figure 5.1).

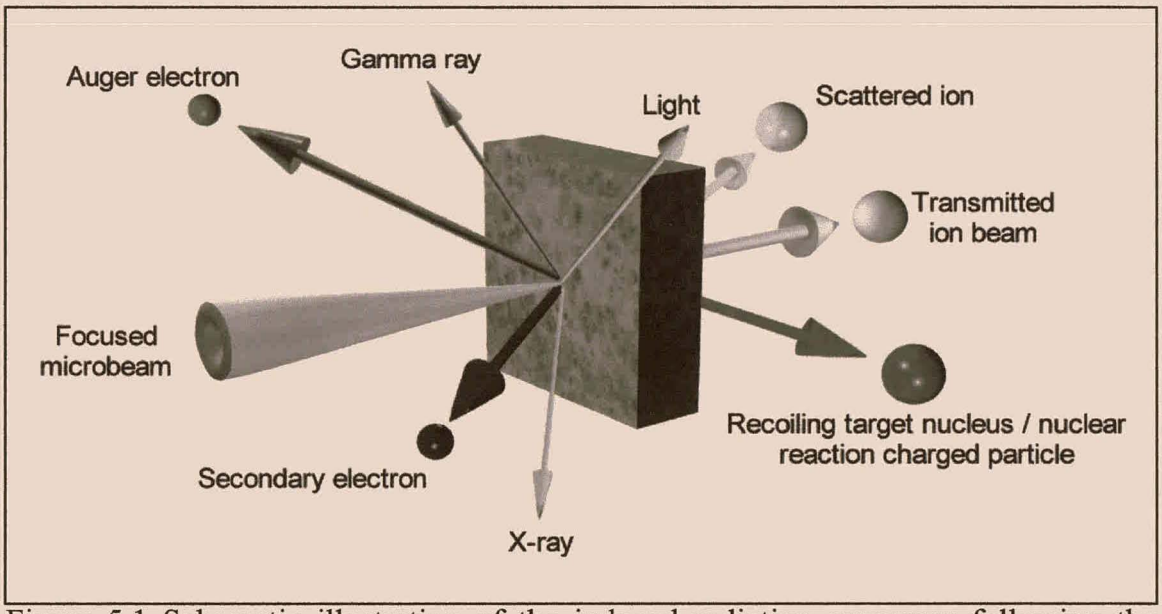

Figure 5.1 Schematic illustration of the induced radiation processes following the impact of a high-energy ion into solid material (adapted from Tapper & Malqvist, 1991)

X-rays are therefore the result of bombarding a specimen with particles so as to eject bound electrons from the *K* or *L* atomic orbits, with the subsequent emission of characteristic X-rays (Cohen, 1990). These *K*- and *L*-X-rays are then detected by means of a Si(Li) X-ray detector, or a High purity germanium (HPGe) detector (Campbell, 1990).

PIXE is a method that uses X-ray emissions for elemental analyses. The major features of PIXE are its multi-elemental character (all elements from boron to uranium can be measured), high sensitivity (absolute detection limits as low as 1 pg, and relative detection limits as low as 0.1 $mg.kg^{-1}$), smooth variation of relative detection limits with atomic number, the ability to analyse small amounts of material (1mg or less), the speed of analyses (can be as low as 1-10 minutes per specimen) and the possibility of automation (Tapper, Przybylowicz & Annegarn, 1994). However, no information relating to the co-ordination chemistry or oxidation state of a particular element can be obtained directly from this technique.

Because of its low detection limits, a major role of the proton microprobe is to extend the quantitative analyses and elemental mapping capability to the $mg.kg^{-1}$ level (Ryan & Jamieson, 1993). In 1995 a rapid matrix transform method called Dynamic analyses was developed for the production of true elemental images using PIXE and the Proton Microprobe (Ryan, Jamieson, Churms & Pilcher, 1995). This system allows for the creation of images that are free of artefacts caused by the overlapping of X-ray lines from different elements, or detector response features of different elements.

The most common use of the PIXE is in the field of geoscience because of its capability for non-destructive in situ microanalyses of fluid inclusions within minerals, and analyses of the elemental composition of mineral grains. For these applications, programs have been developed to increase the accuracy of detection of trace elements down to the limits of detection. This suite of programs is called GeoPIXE (Ryan, Cousens, Sie, Griffin, Suter & Clayton, 1990a; Ryan, Cousens, Sie & Griffin, 1990b) because of its development in the field of geoscience, but they are also useful in the field of biology. Furthermore a PC-based data acquisition system

(VAX) was developed to enable the user to perform a multi-channel analysis or multi elemental mapping (Churms, Pilcher, Springhorn & Tapper, 1993). By using the VAX system together with the GeoPIXE suit of programs, highly accurate multi-elemental analysis can be performed on biological specimens. The micro-PIXE software stores all data relating to the X-ray analyses of elements, so that data of elements not known to be present at the beginning of the run can later be analysed using the stored data.

In the field of biology and medicine, the determination of trace elements in thick biological specimens by means of PIXE is also of importance. The quantification of thick targets is easier than in the case of targets with an intermediate thickness. The reason for this is that it is not important to know the thickness of the target and hence the final proton energy (Tapper *et al.*, 1994). Detection limits are generally slightly higher in comparison to those of thin target analyses, normally in the few $mg.kg^{-1}$ range, but very dependent on the sample matrix and the elements analysed. The sample matrix is an approximation of the major elemental composition of a sample. By using the known composition of 14 biological standards, it was found that a cellulose matrix could be used as representative for all organic materials for elements with $23 \leq Z \leq 56$ with an acceptable error less than 10% (Pineda & Peisach, 1988).

The main limitation in the sensitivity of X-ray techniques is the amount of background. The dominant source of background for the electron probe is Bremsstralung radiation emitted by the electrons from the electron beam undergoing large-angle scattering when "hitting" target electrons. Due to the heavy mass of the ions used for the PIXE technique, the background at lower X-ray energies is mainly attributed to Bremsstralung resulting from secondary electrons ejected by the incident ions (Legge & Cholewa, 1994). Hence, the background in PIXE spectra is normally 2-

3 orders of magnitude lower than for EDAX. Thus the use of high-energy proton beams to excite X-ray production has an advantage over EDAX, because the continuum or Bremsstralung radiation is significantly reduced. The result of the decreased Bremstralung during PIXE, results in the PIXE being capable of detecting elements at the 1 $mg.kg^{-1}$ level, whereas EDAX has a maximum sensitivity in bulk samples of ca.100 $mg.kg^{-1}$.

When entering a specimen the proton beam suffers only slight broadening and penetrates several tens of micrometers into the specimen. An electron beam however, is stopped after 5 – 10 µm and is strongly broadened due to scattering of the electrons (Figure 5.2). Therefore in specimens with a thickness greater than a few micrometers a proton microbeam has a better lateral resolution than an electron microbeam (Johansson & Campbell, 1988). PIXE can therefore be used in the elemental analyses of thick targets, especially in cases where it is difficult or impossible to obtain thin sections. EDAX on the other hand, is essentially a surface layer analyses system, where as PIXE can be used for thin sections, intermediate- and thick specimens.

The micro-PIXE software (GeoPIXE and VAX) stores all X-ray information, the valuable information on trace elements not known to be present at the start of the analyses is therefore not missed later because the experiment can be replayed using the stored data. This is not always possible with current available EDAX software.

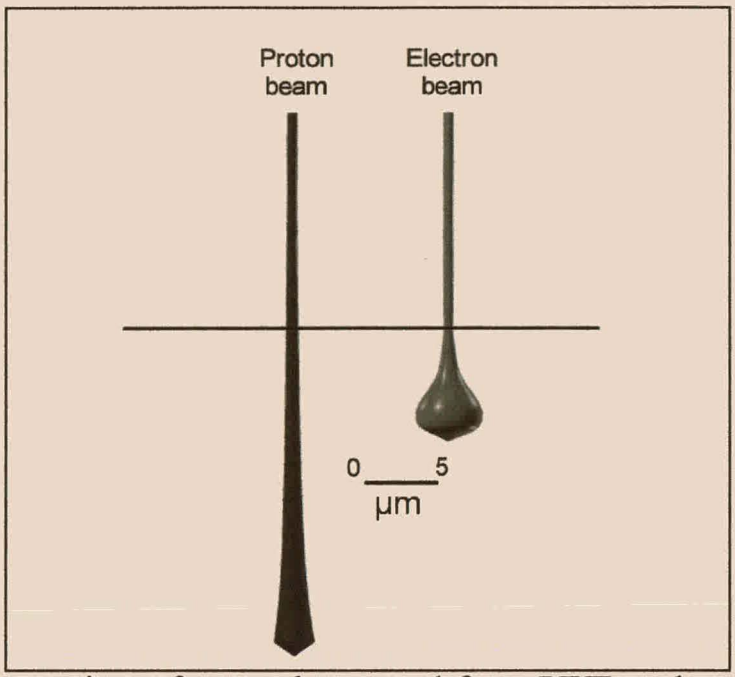

Figure 5.2 Comparison of proton beam used for µ-PIXE analyses and an electron beam in an electron microprobe (Johansson & Campbell, 1988).

5.2.2 Sample preparation:

Before the nephridia of the Cd_{ABS}- and Cd_{PRE}- groups were analysed, an experiment was done to determine whether Cd does accumulate in the nephridia of exposed worms. For this clitellate worms were removed from a culture maintained in the laboratory where $CdSO_4$ has been administered for four years (see section 2.3.1). By the time the worms were removed from this medium, the concentration of Cd in the medium had already built up to more than 300 $mg.kg^{-1}$. Ten worms from this long-term contamination culture were removed and exposed to 300 $mg.kg^{-1}$ $CdSO_4$ in a cattle manure substrate (see section 2.3.3). This substrate had a particle size between 100 and 500 µm, a moisture content of ±70% and a pH of 7 as was previously suggested by Reinecke and Venter (1987). The worms were exposed for the duration of ten days at a temperature of 25°C. Thereafter part of the body wall was dissected

out, together with the nephridia. These samples were then plunge freezed in liquid isopropane cooled down by liquid nitrogen (Echlin, 1992).

After the samples were freezed, they were placed in a CFD Leica freeze dryer and dried over a period of 208 hours, during which time the temperature was raised from -80°C to -40°C. After freeze drying, the samples were carbon coated at the physics department of the University of Stellenbosch. Before and after carbon coating the samples were kept in a dessicator filled with silica gel to keep them dry. The samples were then attached to sample holders designed for the nuclear microprobe. On the samples holders, the samples were attached to a thin film of formvar by means of Araldite, which is a fast setting epoxy adhesive that is chemically pure and does not contain any heavy metals. The samples were then ready for elemental analyses.

To determine whether there would be any differences in the nephridial accumulation of unexposed (Cd_{ABS}-) and historically exposed (Cd_{PRE}-) worms, the same process was carried out on samples of worms originating from both an uncontaminated control, and the long-term Cd-culture. For this experiment, three groups of clitellate worms of the species *E. fetida* originating from cultures maintained in the laboratory, were used (see section 2.3.1). The first group consisted of 10 worms from a control culture, in which no heavy metals had been added to the substrate. This group was used as the unexposed control group (Cd_K). The second group consisted of 20 worms, also originating from a control culture. This group was exposed to $CdSO_4$, thus serving as an exposed control (Cd_{ABS}). The third group also consisted of 20 worms. These worms however originated from a population that had been in a substrate contaminated with cadmium for four years and were used as an exposed experimental group (Cd_{PRE}-).

All three groups were placed in an artificial soil substrate for the duration of 14 days. No $CdSO_4$ was administered to the artificial soil substrate in which the Cd_K-group was placed, while the substrates of the Cd_{ABS}- and Cd_{PRE}- groups was contaminated with $CdSO_4$ to a concentration of 3000 $mg.kg^{-1}$. As shown in Chapter 4, worms that were exposed to high amounts of $CdSO_4$, displayed a disruption in their osmoregulatory function, caused by nephridial degeneration in the affected worms. Therefore, only worms that showed a normal appearance after the exposure period were chosen for PIXE analyses. After the exposure period, samples of the body wall was dissected out from 6 worms of each group, together with the nephridia attached to it, and plunge-freezed in isopropane cooled in liquid nitrogen, freeze dried and carbon coated.

Cd_{ABS}-worms =Worms originating from a culture where they received no exposure to any heavy metals over a period of four years

Cd_{PRE}-worms =Worms originating from a culture where Cd had been administered over a period of four years

Cd_K-worms =Control group, consisting of worms originating from the same culture as the Cd_{ABS}-worms, but that received no exposure during the course of the experiment.

The elemental analyses were performed using the NAC (National Accelerator Centre) nuclear microprobe at Faure, South Africa. This microprobe is based on a 6 MeV single ended Van de Graaff accelerator and uses Oxford Microprobe triplet lenses for beam focusing (Prozesky, Przybylowicz, Van Achterbergh, Churms, Pineda, Springhorn, Pilcher, Ryan, Kritzinger, Schmitt & Swart, 1995). Resolutions of the order of 1 μm beam spot are achievable for a proton current of 100 pA. In the present study a 3 MeV proton beam was used, focused to about 3 x 3 μm^2 and scanned over areas from about 1.5 x 1.5 mm down to 60 x 60 μm on selected structures. The current was kept at about 400 pA to minimise beam damage and this resulted in count rates of the order of 500 c/s. The total accumulated charge for the scanned areas varied between 300 nC and 20 μC. PIXE spectra were collected using PGT Si(Li) X-ray detector of 30 mm^2 active area and 8 μm Be window, positioned at 135°, about 30mm from the target. A 150 μm Kapton filter was used to shield the detector from backscattered protons and to reduce the intensity of X-rays from major, light elements. Spectra were analysed using the GeoPIXE suite of programs (Ryan & Jamieson, 1993; Ryan *et al.*, 1995). Only thick targets were analysed and their matrix composition was approximated by that of cellulose (Pineda & Peisach, 1988).

Elemental maps were obtained using a rapid matrix transform method called Dynamic Analysis (DA), forming part of the GeoPIXE package (Ryan & Jamieson, 1993; Ryan *et al.*, 1995). The images are inherently overlap-resolved, background subtracted and the maps are generated on-line. The final maps give quantitative elemental images with the intensity in $mg.kg^{-1}$. The procedure of its use in biological studies was described by Przybylowicz, Pineda, Prozesky & Mesjasz-Przybylowicz (1995), and Przybylowicz, Mesjasz-Przybylowicz, Pineda, Churms, Springhorn &

Prozesky (1999). Scanned regions were divided into 64 x 64 pixels, with scanning speed 10 ms/pixel, and the final maps were contours linking pixels with similar values. They were obtained using programs written in the Interactive Data Language.

Both *K* and *L*-X-ray lines were considered for mapping Cd distribution. In collected spectra *K*-X-ray lines of this element were free of overlap interference, but these lines are weak due to unfortunate combination of relatively low *K*-shell ionisation cross-sections and the deteriorating efficiency of a Si(Li) detector at X-ray energies above 20 keV. The presence of potassium at 1 wt.% level makes Cd analyses based on *L*-X-ray lines difficult and not reliable even when using full non-linear least-squares method at found Cd levels between 70 and 900 ppm. The ratio of peak areas of major lines Cd-*L*/potassium-*K* varies between only 0.7 – 1% and all Cd *L*-X-ray lines overlap with either potassium *K*-X-ray lines or with their low energy tails. Therefore, based on minimum limits of detection and error estimates of obtained concentrations (Table 5.1), the analyses based on Cd *K*-X-ray lines were used as the only option, both for mapping using Dynamic Analysis and for evaluating the concentrations in selected, smaller areas. Concentrations obtained using full non-linear least-squares method from these areas were compared with results from elemental mapping.

5.3 Results

Figure 5.3 shows a part of the body wall, with the nephridia attached, prior to freezing (Figure 5.3A), after plunge freezing of the sample in liquid isopropane and before analysis (Figure 5.3B) and the same sample after analyses (Figure 5.3C.) When the samples were plunge frozen, a layer formed that obscured the structure of the nephridia, but did not seem to influence the analysis of the elements within the sample. This layer was probably the result of an interaction of the isopropane with the mucus surrounding the tissue. The area analysed and represented in Figure 5.4 and Figure 5.5 are indicated in Figure 5.3 C by the large and smaller white squares. The areas where spot analyses were conducted (values represented in Table 5.1) can also be seen as little black squares indicated by arrows.

From the X-ray mapping (Fig. 5.4 and Fig. 5.5) it can be seen that Cd did accumulate in the nephridia of the earthworms exposed to the metal. It also showed a distribution pattern in the nephridium itself. The highest accumulation was in the region stretching from the nephrostome to the first loop (Table 5.1). Concentrations from small analysed areas of different regions of the body wall and nephridium showed the following distributions. The lowest concentration was found in the body wall 76 ± 15 $mg.kg^{-1}$ (Figure. 5.4 A). The region of the second loop followed with a concentration of 240 ± 40 $mg.kg^{-1}$ (Figure. 5.4 B). The second highest concentration was observed in the urinary vasiculus, 570 ± 21 $mg.kg^{-1}$ (Figure. 5.4 C), and the highest in the area between the nephridiopore and the first loop, 890 ±30 $mg.kg^{-1}$ (Figure. 5.4 D). These values are represented in Table 5.1.

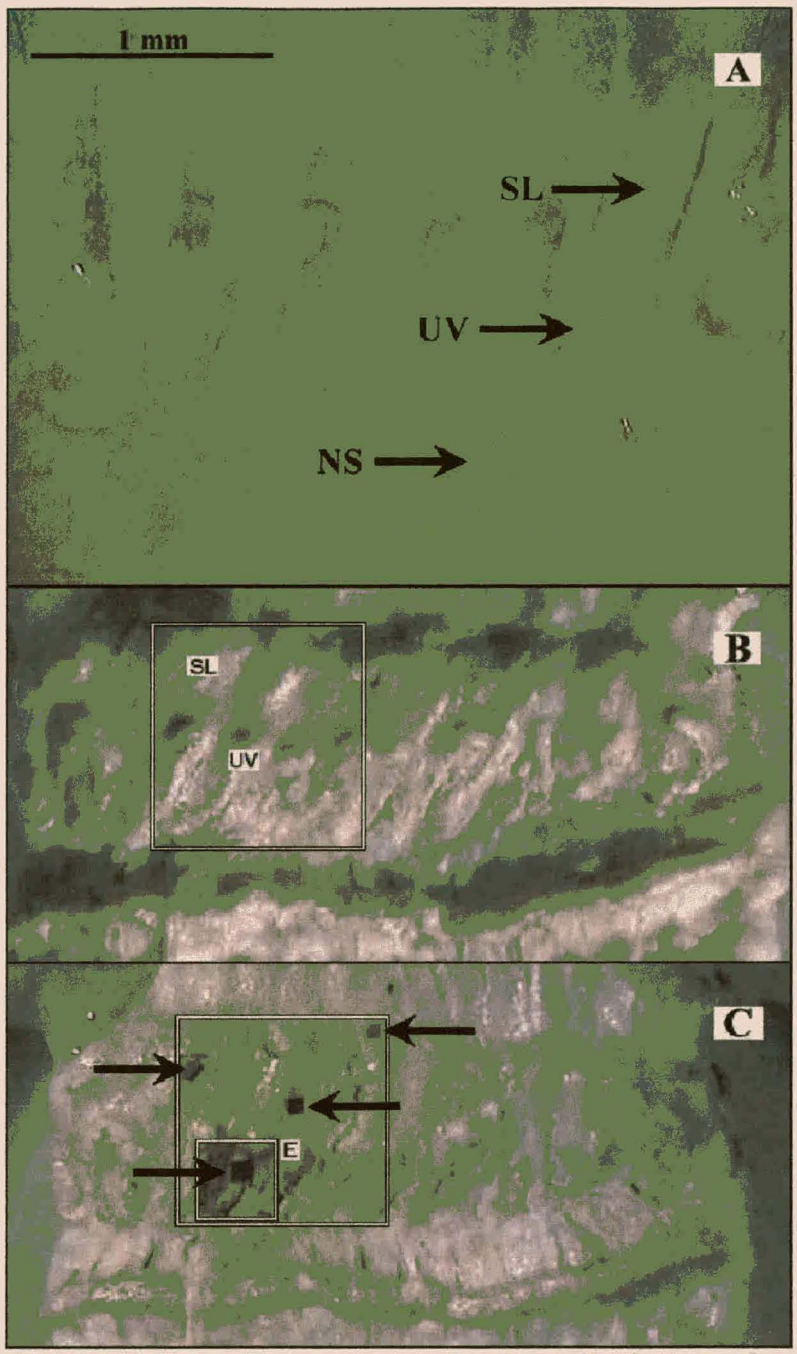

Figure 5.3 Micrographs of (A) A sample of the body wall of the earthworm of the species *Eisenia fetida* (Oligochaeta) containing the nephridia, (B) a freeze-dried specimen of the before PIXE analyses, and (C) the same sample after PIXE analyses. Beam damage allows easy localisation of the measured areas; the bigger square indicates the scanned region represented in Figure 5.4, covering an area of 1.5 x 1.5 mm, and the smaller square (E) indicates smaller scanned region represented in Figure 5.5. The four small black squares indicated by the arrows (C), are the regions where small area analyses (represented in Table 5.1) were conducted. The following regions of the nephridia are also indicated; the nephrostome (NS), the urinary vasiculus (UV), and the second loop (SL).

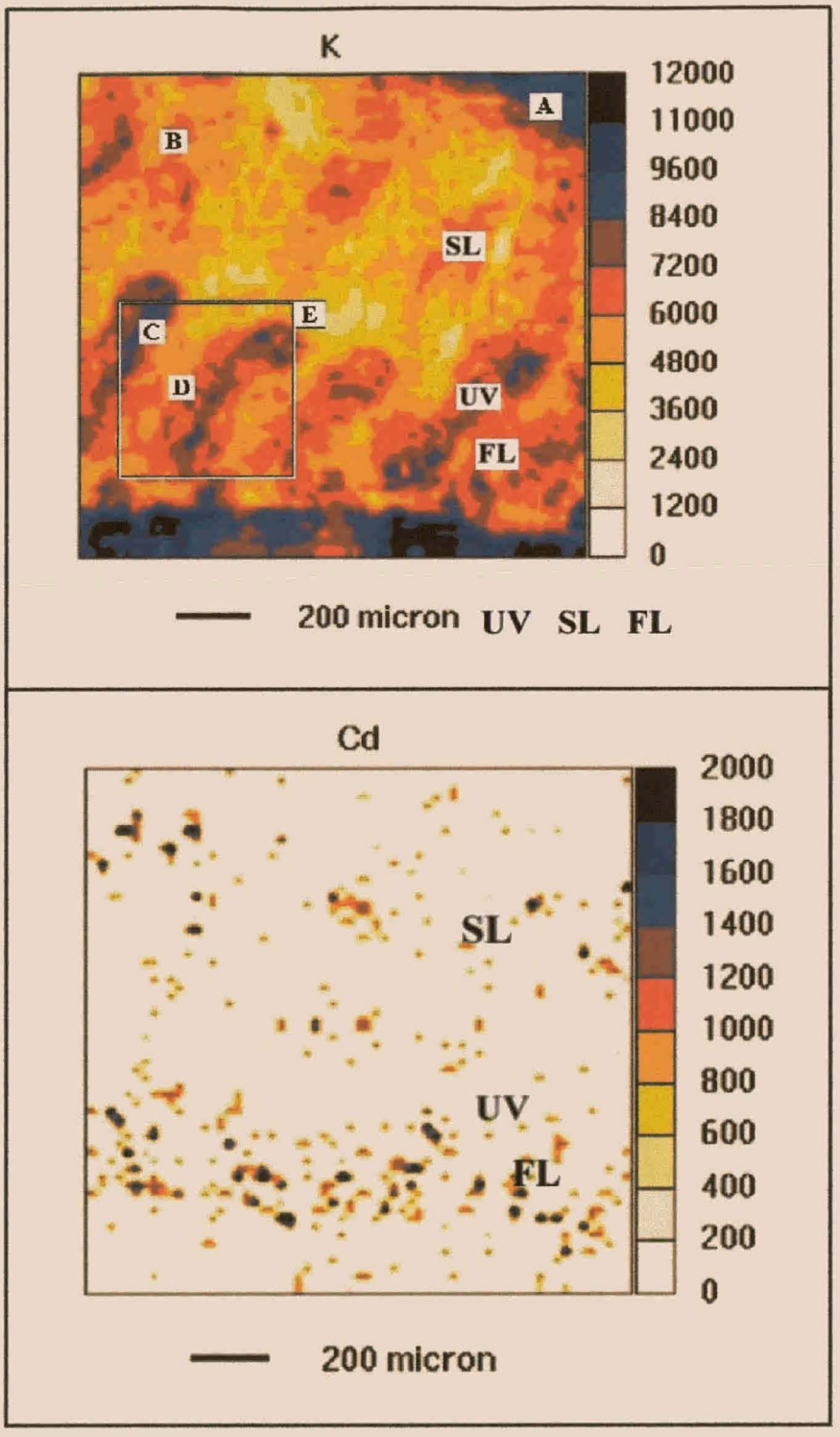

Figure 5.4 Elemental maps of K and Cd and in the nephridium of the earthworm. *Eisenia fetida* (Oligochaeta). A, B, C and D indicate small areas where additional analyses were performed (Table 5.1). The square (E) indicates the area represented by Figure 5.5. The following regions of the nephridia are also indicated; the nephrostome (NS), the urinary vasiculus (UV), and the second loop (SL). Image intensity in $mg.kg^{-1}$.

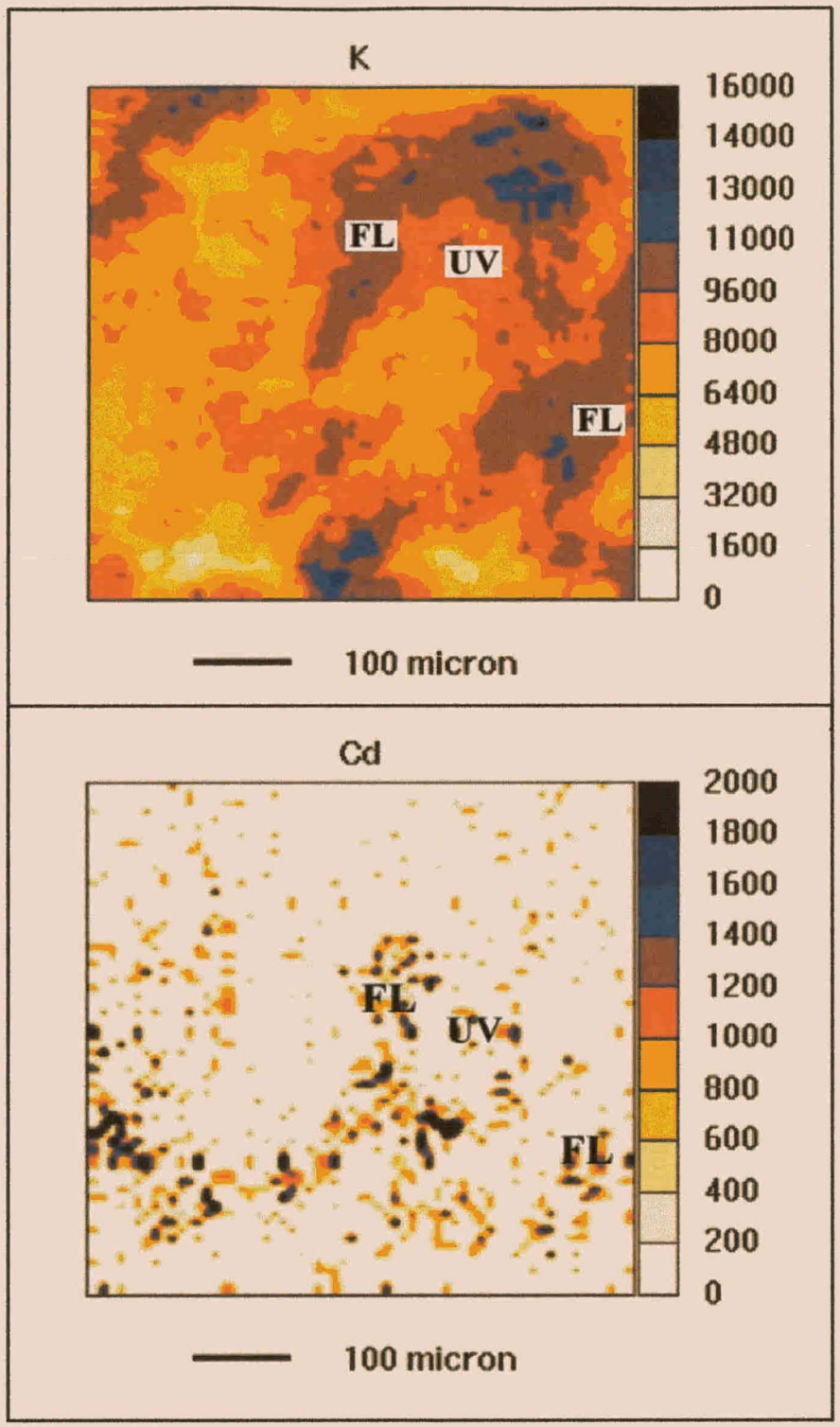

Figure 5.5 Elemental maps of K and Cd in the region of the nephridia closest to the ventral nerve cord to show more clearly the extensive accumulation of Cd in the region between the nephrostome and first loop. The following regions of the nephridia are also indicated; the nephrostome (NS), the urinary vasiculus (UV), and the second loop (SL). Image intensity in $mg.kg^{-1}$.

Table 5.1. Cd concentrations obtained using full non-linear least-squares method (used by the GeoPIXE suite of programs) in selected areas of the earthworm *Eisenia fetida* (Oligochaeta). All values are in $mg.kg^{-1}$ ($\pm$ 1σ uncertainty). Values in brackets are minimum detection limits (99% confidence level). Note high errors and detection limits for Cd when using *L*-X-ray lines, due to overlaps with Potassium *K*-X-ray lines. Potassium concentrations and the area ratio of major interfering lines are also shown.

	Area ratio (Major lines) Potassium *K*/Cd*L*	Potassium	Cd*L*	Cd*K*	Accumulated charge
	[%]	Values in $mg.kg^{-1}$			μC
Reference spectrum for DA mapping	0.91	8170 ± 150 (8.5)	330 ± 160 (33.4)	96 ± 23 (16.7)	1.143
Total amount of Cd for the area represented in Figure 5.4	0.8	8240 ± 150 (4.0)	280 ± 140 (12.0)	140 ± 8 (6.7)	4.751
Total amount of Cd for the area represented in Figure 5.5	1.12	11000 ± 210 (9.0)	560 ± 240 (17.0)	250 ± 14 (7.3)	5.370
Body wall (Figure 5.4 A)	0.7	12520 ± 240 (15.2)	400 ± 4400 (1180)	76 ± 15 (10.1)	1.003
Second loop (Figure 5.4 B)	1.06	10930 ± 210 (17.5)	520 ± 240 (30.1)	240 ± 40 (23.4)	0.832
Urinary vasiculus (Figure 5.5 C)	1.3	14150 ± 290 (10.0)	830 ± 15300 (1330)	570 ± 21 (6.0)	6.402
Area between the nephridiopore and first loop (Figure 5.4 D)	1.8	13270 ± 270 (14.4)	1070 ± 330 (23.0)	890 ± 30 (10.9)	3.027

For comparison between the worms that had a history of Cd exposure (Cd_{PRE}-), and the worms that had no prior exposure (Cd_{ABS}-) analyses were conducted on the nephridia of six individuals of each group. The analyses were conducted on the areas of the nephridia with the highest (region of first loop) and lowest (area of second loop) Cd concentration. In both cases there was a statistically significant difference ($P < 0.001$) between these two regions. This means that the nephridia of both groups accumulated more Cd in the proximal region of the nephridia than in the region of the second loop. Comparison of the Cd-concentrations in the proximal region of the nephridia from the Cd_{ABS}- and Cd_{PRE}-worms showed a statistically significant difference ($P < 0.001$) between the two groups, with the highest accumulation present in the proximal region of the Cd_{PRE}-worms (Table 5.2).

There was also a statistically significant difference ($P < 0.001$) between the two groups in relation to the values of accumulated Cd within the second loop, with the Cd_{PRE}-worms again displaying the highest accumulation (Table 5.2). The only values between the two groups that did not show a statistically significant difference, was that between the accumulated values in the region of the second loop of the Cd_{PRE}-worms, and that of the region of the first loop of the Cd_{ABS}-worms ($P > 0.001$). The averages of accumulation of cadmium within these groups are presented in Table 5.2.

Table 5.3 shows the concentrations of Cd and sulphur in the regions of the first loop, second loop, body wall, and the area between the nephridiopore and first loop, as well as the total concentration of Cd and sulphur for the area's represented in figure 5.4 and 5.5.

Table 5.2. Concentration of cadmium achieved by small area analyses. The concentrations were detected on the *K*-X-ray lines, as they proved more reliable than the *L*-K-ray lines. The concentrations are represented in $mg.kg^{-1}$.

	Control (n=6)	Cd_{ABS}-FL (n=6)	Cd_{ABS}-SL (n=6)	Cd_{PRE}-FL (n=6)	Cd_{PRE}-SL (n=6)
Mean	0	836.33	250.83	2195.00	406.17
STD DEV	0	600.38	84.41	828.03	173.70

Cd_{ABS}-FL = small area analyses in the region of the first loop of worms with no prior history of exposure to Cd.

Cd_{ABS}-SL = small area analyses in the region of the second loop of worms with no prior history of exposure to Cd.

Cd_{PRE}-FL = small area analyses in the region of the first loop of worms that had a prior history of exposure to Cd.

Cd_{PRE}-SL = small area analyses in the region of the second loop of worms that had a prior history of exposure to Cd.

Table 5.3 Cd- and sulphur concentrations obtained using full non-linear least-squares method (used by the GeoPIXE suite of programs) in selected areas of the earthworm *Eisenia fetida* (Oligochaeta). All values are in $mg.kg^{-1}$ ($\pm$ 1σ uncertainty). Values in brackets are minimum detection limits (99% confidence level).

	Area ratio (Major lines) Potassium *K*/Cd*L*	Sulphur	Cd*L*	Cd*K*	Accumulated charge
	[%]	Values in $mg.kg^{-1}$			µC
Reference spectrum for DA mapping	0.91	2477 ± 177 (151)	335 ± 162 (33.4)	95.7 ± 23 (16.7)	1.143
Total amount of Cd for the area represented in Figure 5.4	0.8	1382 ± 168 (45.7)	283 ± 138 (12.0)	141 ± 7.7 (6.7)	4.751
Total amount of Cd for the area represented in Figure 5.5	1.12	2117 ± 286 (102)	558 ± 238 (17.0)	252 ± 13.9 (7.3)	5.370
Body wall (Figure 5.4 A)	0.7	204 ± 101 (97.6)	404 ± 4412 (1179)	75.8 ± 14.5 (10.1)	1.003
Second loop (Figure 5.4 B)	1.06	1290 ± 243 (118)	523 ± 244 (30.1)	237 ± 41 (23.4)	0.832
Urinary vasiculus (Figure 5.5 C)	1.3	811 ± 153 (45.6)	833 ± 15270 (1325)	568 ± 20.7 (6.0)	6.402
Area between the nephridiopore and first loop (Figure 5.4 D)	1.8	894 ± 297 (140)	1070 ± 335 (22.9)	887 ± 32.6 (10.9)	3.027

5.4 Discussion

From the X-ray mapping it was seen that the cadmium was accumulated and restricted to the greatest extent in the first part of the nephridium consisting of the nephrostome and the first series of convoluted tubules up to, and including the region of the first loop. This seems to correlate to findings in humans where renal accumulation of cadmium is restricted to the cortex of the kidney (Elinder, 1977). The glomeruli as well as the proximal- and distal convoluted tubuli are situated in the cortex of the human kidney (Guyton, 1991). The glomerulus of the mammalian kidney is analogous in function to the nephridiopore of the earthworm nephridia. It might be possible that the first and second loops of the earthworm nephridia are also analogous in function to the proximal- and distal convoluted tubuli found in the mammalian kidney. However, in the earthworm nephridium the highest accumulation takes place in the first loop and the lowest, in the region of the second loop. This seems to be contradictory to what is found in the mammalian kidney where accumulation takes place in the cortex, where both the first and second convoluted tubuli are situated. The distribution found in the renal cortex of mammalian kidneys however, does not reflect the tubular accumulation patterns, but an averaged accumulation since the individual tubules in the renal cortex are interlaced with one another. In the mammalian kidney the proximal tubules are the ones primarily affected by Cd (Ishido *et al.*, 1998). It is therefore not surprising that in the earthworm nephridia the highest accumulation takes place in the region of the first loop. This may well be the case in the mammalian kidney as well, but this distinction might be obscured by the complex structure of the renal cortex.

From the PIXE analyses of the exposed nephridia, no correlation between sulphur and cadmium was observed (Table 5.3). This would suggest that the Cd accumulated within the nephridia was not bound to a metallothionein, the reason being that metallothioneins contain high amounts of the amino acid cysteine which in turn contains sulphur. This may be an indication of a mechanism similar to the one suggested by Ishido *et al.* (1998) in mammals, where Cd is bound by a metallothionein and transported to the proximal renal tubuli cells, in which Cd-metallothionein complex dissociates and the free Cd^{2+} is released within the cells. These authors suggest that this free intracellular Cd^{2+} cause biochemical disruptions leading to cellular damage. If indeed the earthworm nephridium reacts to Cd in the same way a mammalian nephron does, it would be expected (as the results indicate) that highest accumulation of Cd would be found in the region of the first loop, which is comparable to the proximal convoluted tubule of the mammalian nephron.

As to the differences displayed in the accumulation of Cd within certain regions of the nephridia of Cd_{PRE}- and Cd_{ABS}-worms, it was found that Cd_{PRE}-worms showed a substantial higher accumulation, both in the region of the first loop, and in the region of the second loop. This may indicate that these worms possess some kind of mechanism, which allows their nephridia to concentrate higher amounts of cadmium than is the case for Cd_{ABS}-worms. This might explain the differences in cell percentages observed between the bloated (^{B}Cd-) worms of the Cd_{ABS}- and Cd_{PRE}-groups in the previous chapter. It was found that Cd_{PRE}-worms, which showed a disruption in osmolality as a result of water retention, caused by nephridial degeneration as a result of acute exposure to high $CdSO_4$ concentrations, still maintained a normal cell percentage (Table 5.1). However, worms from the control group (Cd_{ABS}-) that received the same treatment and showed the same disruption in

osmoregulatory capacity, showed a decrease in the cell percentage of the coelomic fluid. It may be possible that due to the historic exposure of the Cd_{PRE}-worms, there has developed some mechanism that not only allows the nephridia of these worms to accumulate Cd to a higher extent, as shown in Table 5.2, but that the nephridia of these worms are capable of removing enough Cd from the coelomic fluid so as to lower the Cd concentration of the coelomic fluid to a level that allows coelomocite survival.

CHAPTER 6

Conclusion and summary

From the life history experiments where the percentage changes in biomass over an eight week period was monitored, a clear indication of a Cd-dependency was observed in the Cd_{PRE}-worms when placed in an uncontaminated substrate. This was characterised by an initial increase in the mean percentage biomass change for the first four weeks, whereafter the worms displayed a negative change in biomass. This negative trend continued from the fifth week until the termination of the experiment after eight weeks.

The cocoon production of the Cd_{PRE}-worms in substrates contaminated to different degrees, showed a trend that can be associated with the development of an increased resistance to Cd, as well as a Cd-dependency. In the uncontaminated substrate these worms produced less cocoons than in the moderately contaminated substrate, and the lowest cocoon production occurred in the substrate with the highest concentration of $CdSO_4$.

When comparing the percentages of viable cocoons it was found that in all three substrates ranging from an uncontaminated control, to a highly contaminated substrate, the Cd_{PRE}-groups had a higher percentage of viable cocoons than the Cd_{ABS}-groups. This might be an indication of some kind of mechanism present in the Cd_{PRE}-worms enabling them to overcome, or reduce the effects of sperm damage usually associated with heavy metal exposure.

On a physiological level, worms of both the Cd_{ABS}- and Cd_{PRE}-groups that were affected detrimentally by high concentrations of $CdSO_4$ in the substrate (bloated

worms), displayed the same decrease in coelomic osmolality. There was however a difference between the bloated Cd_{ABS}- and Cd_{PRE}- groups when the cell percentages of the coelomic fluid were compared. The $^{B}Cd_{PRE}$-worms retained a normal cell percentage, while the $^{B}Cd_{ABS}$-worms showed a sharp drop in the cell percentage of the coelomic fluid. This could be an indication of either a cellular resistance of coelomocytes, or the presence of an effective mechanism to reduce the Cd content of the coelomic fluid to levels conducive to cell survival in the Cd_{PRE}-worms. Histologically no differences in the degree of nephridial damage between worms of the two groups displaying the same level of osmotic disruption, were observed.

Nephridial accumulation of Cd was higher in both the proximal- (region up to the first loop) and distal region (region of the second loop) of the nephridia of the Cd_{PRE}-groups. This difference was so high that the lowest concentration of Cd in the Cd_{PRE}-nephridia correlated to the highest concentrations found in the Cd_{ABS}-nephridia. This indicates that the Cd_{PRE}-worms can accumulate Cd to a much higher concentration, and may even be able to extract enough Cd from the coelomic fluid, to explain why the percentage of coelomocytes in the bloated Cd_{PRE}-worms did not decrease as was the case in the bloated Cd_{ABS}-worms. From the life history experiments it was shown that at sublethal $CdSO_4$ concentrations, there were no differences between the Cd_{ABS}- and Cd_{PRE}-groups, in total body accumulation of Cd after the eight-week exposure period. However, given the fact that the nephridia of the Cd_{PRE}-group accumulated Cd to a much higher extent than those of the Cd_{ABS}-group, there might be a difference in the distribution pattern of the accumulated Cd within the body compartments of the Cd_{PRE}-Worms, as opposed to the and Cd_{ABS}-worms. For example, the Cd_{PRE}-group might be able to concentrate the Cd efficiently in the nephridia and cloragogenous tissue, to reduce the detrimental effects of the Cd on the

spermatozoa. This could explain the high percentages of viable cocoons produced by the Cd_{PRE}-groups at different concentration levels of $CdSO_4$.

To conclude, definite indications of a developed increase in resistance to $CdSO_4$ were observed in the pre-exposed worms, as indicated in Table 6.1. Whether this increased resistance has occurred as a result of acclimation, or a genetically based tolerance, can not be determined from this study. It can only be concluded that there is a definite indication of an increased resistance in the pre-exposed group. Further studies should be conducted on the F1 and F2 generations of the pre-exposed groups so as to determine whether the observed resistance is the result of a genetic adaptation to the polluted environment, or not.

Table 6.1 Summary of the results achieved from the different parameters investigated.

Parameter Investigated	**Result**
Percentage change in biomass	Indication of Cd dependency in the Cd_{PRE}-group.
Cocoon production	Clear sign of resistance in Cd_{PRE}-group
Percentage viable cocoons	Cd_{PRE}-groups have higher percentage viable cocoons in all cases
Worms produced per cocoon	No differences between Cd_{ABS}- and Cd_{PRE}-groups
Body concentration of Cd	No differences between Cd_{ABS}- and Cd_{PRE}-groups after exposure
Osmolality of coelomic fluid after exposure to high concentrations of $CdSO_4$	No differences between Cd_{ABS}- and Cd_{PRE}-groups

Cell percentages in coelomic fluid after exposure to high concentrations of $CdSO_4$	Bloated Cd_{PRE}-worms do not show a decrease in cell percentage, while bloated Cd_{ABS}-worms show a definite decrease
Histological evaluation of nephridia after exposure to high concentrations of $CdSO_4$	Cd_{ABS}- and Cd_{PRE}-worms show same degree of nephridial damage for both bloated and normal appearing worms
Cd accumulation in proximal region of the nephridia (up to first loop)	Cd_{PRE}-worms show much higher accumulation of Cd in this region than Cd_{ABS}-worms
Cd accumulation in distal region of the nephridia (area of second loop)	Cd_{PRE}-worms again show much higher accumulation of Cd in this region than Cd_{ABS}-worms

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APPENDIX 1

Mallory-Heidenhain aniline blue staining method

MALLORY-HEIDENHAIN'S ANILINE BLUE STAIN (AZAN STAIN)

INDICATIONS. Stain for alpha cells of pancreas
Stain for collagen

FIXATION. Zenker's, Helly's, Bouin's, or Carnoy's

TECHNIQUE. Paraffin

Solutions

AZOCARMINE G SOLUTION

Azocarmine G	1 to 1.5	gm.
Distilled water	200.0	ml.

Bring to boil. Filter through coarse filter paper in paraffin oven at 58°C so that fine particles of dye (needle-like crystals) will also pass through. When cool, add 2 ml. of glacial acetic acid. Keep in refrigerator. Filter before use.

Note: Azocarmine B has been replaced with Azocarmine G because of the latter's greater solubility and staining power.

ANILINE-ALCOHOL SOLUTION

Aniline oil	1ml
Alcohol, 95%, ethyl	100 ml.

1% GLACIAL ACETIC ALCOHOL

Glacial acetic acid	1 ml.
Alcohol, 95%, ethyl	100 ml.

5% PHOSPHOTUNGSTIC ACID SOLUTION

Phosphotungstic acid	5 gm.
Distilled water	100 ml.

STOCK ANILINE BLUE SOLUTION

Aniline blue, water soluble	0.5 gm.
Orange 0	2.0 gm.
Distilled water	100.0 ml.
Glacial acetic acid	8.0 ml.

WORKING ANILINE BLUE SOLUTION

Aniline blue stock solution	1 part
Distilled water	2 parts

Staining Procedure

1. Deparaffinize sections through xylene, absolute and 95% alcohols down to water. Remove mercuric chlorides with iodine, followed by hypo, if necessary.
2. Rinse in distilled water.
3. Stain in azocarmine C solution in a covered dish in an oven at 58°C for ***15 to 20 minutes***. Allow to cool for ***5 minutes*** at room temperature.
4. Rinse in distilled water.
5. Differentiate in the aniline-alcohol solution until cytoplasm and connective tissue are pale pink and nuclei stand out sharply. Control differentiation by rinsing slide in 1% glacial acetic alcohol and check with microscope. If section is too red, return to aniline-alcohol. Rinse with 1% glacial acetic alcohol.
6. Mordant in 5% phosphotungstic acid solution until the connective tissue is completely decolourised ***15 minutes*** **to** ***1 hour***. Check with microscope at 15-minute intervals.
7. Rinse quickly in distilled water.
8. Counterstain from ***5*** **to** ***30 minutes*** in the working aniline blue solution until the finest connective tissue fibers are sharply stained. Examine from time to time with microscope.
9. Rinse in distilled water.
10 Dehydrate quickly through 95% alcohol, two changes of absolute alcohol.
11. Clear with two or three changes of Xlyene, and mount in Permount.

RESULTS.	Chromatin and neuroglia	red
	Cytoplasm	pink to blue
	Collagen and reticulum	blue
	Muscle	red to orange

REFERENCE. Mallory, F. B. *Pathological Technique*. Philadelphia: Saunders, 1938, pp. 154.

APPENDIX 2

Experimental data

Cd_{ABS}-worms

[$CdSO_4$] in the substrate = 0 $mg.kg^{-1}$

	Beginning	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
1	0.3087	0.3553	0.3906	0.3906	0.3844	0.3481	0.3102	0.2025	
2	0.3517	0.4427	0.4156	0.4156	0.4202	0.3658	0.3333	0.3588	0.3103
3	0.3613	0.4622	0.4325	0.4325	0.438	0.4072	0.3437	0.4002	0.3263
4	0.3822	0.4764	0.4533	0.4533	0.4408	0.4233	0.4114	0.4895	0.4202
5	0.3955	0.4966	0.4683	0.4683	0.4954	0.4254	0.4253	0.4923	0.4311
6	0.401	0.5006	0.4752	0.4752	0.5021	0.435	0.4272	0.4936	0.4415
7	0.4083	0.5102	0.4986	0.4986	0.5023	0.4444	0.4449	0.5232	0.4432
8	0.4093	0.5139	0.5158	0.5158	0.507	0.447	0.4506	0.5414	0.4573
9	0.4098	0.5312	0.5196	0.5196	0.5076	0.4478	0.4513	0.5436	0.4619
10	0.4197	0.5632	0.5487	0.5487	0.5134	0.4626	0.46	0.5479	0.4752
11	0.4567	0.5721	0.5678	0.5678	0.5344	0.472	0.4665	0.5653	0.4824
12	0.4756	0.5803	0.5738	0.5738	0.5413	0.4858	0.5058	0.6053	0.5104
13	0.489	0.5809	0.5801	0.5801	0.5434	0.5161	0.5065	0.6078	0.5109
14	0.5052	0.5897	0.5874	0.5874	0.5716	0.5448	0.5325	0.656	0.5571
15	0.5123	0.6001	0.5886	0.5886	0.5792	0.5523	0.5406	0.6764	0.5804
16	0.5215	0.6092	0.6094	0.6094	0.6103	0.5603	0.5881	0.7222	0.6129
17	0.5369	0.6122	0.6335	0.6335	0.6124	0.5672	0.6033	0.7606	0.6459
18	0.5532	0.6672	0.7033	0.7033	0.6633	0.6525	0.6457	0.7662	0.6775
19	0.5671	0.7227	0.7347	0.7347	0.7174	0.6752	0.6654	0.8161	0.6876
20	0.596	0.748	0.7378	0.7378	0.7177	0.7015	0.6764	0.8679	0.691

AVG	0.45305	0.556735	0.55173	0.55173	0.54011	0.496715	0.489435	0.58184	0.511742
STDEV	0.0797034	0.093733	0.10043	0.10043	0.091051	0.098373	0.106679	0.161726	0.113692

Cd_{ABS}-worms

[$CdSO_4$] in the substrate = 600 $mg.kg^{-1}$

	Beginning	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
1	0.3089	0.1636	0.1837	0.179	0.2621	0.2738	0.2991	0.2996	0.2735
2	0.3095	0.3268	0.3703	0.3414	0.3918	0.3786	0.3833	0.349	0.2792
3	0.3106	0.351	0.3886	0.3542	0.4016	0.4446	0.4313	0.4037	0.3832
4	0.3352	0.3763	0.4196	0.3844	0.4136	0.4707	0.4415	0.4368	0.3922
5	0.3552	0.3829	0.4226	0.3962	0.4628	0.4714	0.4536	0.4461	0.4095
6	0.3674	0.3877	0.4334	0.4022	0.4804	0.48	0.473	0.4484	0.4125
7	0.3749	0.3912	0.4548	0.4063	0.4907	0.481	0.5087	0.4629	0.4291
8	0.3757	0.4249	0.4621	0.4283	0.4994	0.4944	0.5143	0.4647	0.4381
9	0.3885	0.4311	0.4673	0.4314	0.5085	0.5558	0.5156	0.4814	0.4568
10	0.3943	0.4468	0.4691	0.4537	0.5249	0.559	0.5374	0.4879	0.4934
11	0.3987	0.4673	0.5077	0.4613	0.5525	0.56	0.5385	0.5343	0.5114
12	0.4197	0.4741	0.5323	0.4672	0.5606	0.5688	0.556	0.5345	0.5192
13	0.4201	0.4905	0.5334	0.4851	0.5805	0.5722	0.5776	0.5418	0.5194
14	0.4304	0.5086	0.5337	0.5106	0.6056	0.5752	0.6051	0.5747	0.5361
15	0.4502	0.511	0.5392	0.5113	0.6075	0.63	0.6375	0.5925	0.5528
16	0.4716	0.5239	0.5517	0.5355	0.6521	0.64	0.6456	0.5992	0.5703
17	0.4802	0.5299	0.5833	0.5364	0.6565	0.6461	0.6697	0.6131	0.5981
18	0.4907	0.5308	0.5861	0.5367	0.6571	0.6635	0.6766	0.6265	0.6198
19	0.5108	0.5688	0.5949	0.5583	0.6598	0.6651	0.6818	0.6814	0.6267
20	0.5665	0.6151	0.6119	0.5719	0.6757	0.6763	0.7352	0.7028	0.6341

AVG	0.407955	0.445115	0.482285	0.44757	0.532185	0.540325	0.54407	0.514065	0.474805
STDEV	0.071042	0.101104	0.099694	0.093043	0.110409	0.104537	0.111975	0.1056	0.106107

Cd_{ABS}-worms

[$CdSO_4$] in the substrate =1200 $mg.kg^{-1}$

	Beginning	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
1	0.3113	0.3078	0.2839	0.2401	0.3194	0.2778	0.2541	0.2299	0.1863
2	0.3231	0.3404	0.3627	0.3108	0.333	0.3155	0.2598	0.2407	0.1889
3	0.3347	0.349	0.3674	0.3163	0.3579	0.3282	0.3379	0.3125	0.2598
4	0.3463	0.3513	0.3828	0.3229	0.3602	0.3415	0.3483	0.3269	0.2858
5	0.3497	0.3658	0.3876	0.3264	0.3662	0.3628	0.4	0.3684	0.3064
6	0.3515	0.3753	0.3884	0.331	0.37	0.3776	0.4013	0.3835	0.3149
7	0.359	0.3776	0.3887	0.3315	0.3925	0.3871	0.4055	0.388	0.3244
8	0.3828	0.3985	0.3936	0.3315	0.4057	0.3914	0.4145	0.4038	0.345
9	0.3863	0.4058	0.3964	0.3338	0.4066	0.3917	0.4183	0.4356	0.3708
10	0.3874	0.4154	0.4033	0.3501	0.4187	0.4174	0.4227	0.4429	0.3838
11	0.4098	0.4159	0.4301	0.3952	0.4364	0.4209	0.4358	0.4513	0.4229
12	0.4103	0.4265	0.4528	0.4068	0.4553	0.4501	0.4802	0.4902	0.4668
13	0.4131	0.4463	0.4562	0.4077	0.4581	0.4584	0.4914	0.5169	0.4757
14	0.4142	0.447	0.4587	0.4144	0.4815	0.4851	0.5073	0.5579	0.5048
15	0.4153	0.462	0.4634	0.4331	0.4986	0.4942	0.5181	0.558	0.5267
16	0.4169	0.4628	0.4771	0.437	0.5057	0.5001	0.5453	0.5963	0.5648
17	0.4381	0.4671	0.5097	0.439	0.5449	0.5394	0.566	0.6147	0.5661
18	0.4514	0.4908	0.5117	0.4578	0.5455	0.5767	0.5735	0.6157	0.5775
19	0.4945	0.5291	0.5193	0.4787	0.5918	0.5818	0.5796	0.6234	0.5938
20	0.5023	0.5631	0.5487	0.4991	0.6036	0.6011	0.606	0.6612	0.5947

AVG	0.3949	0.419875	0.429125	0.37816	0.44258	0.43494	0.44828	0.46089	0.403432
STDEV	0.052415	0.065209	0.065237	0.067686	0.0847	0.093294	0.101809	0.130043	0.134729

Cd_{PRE}-worms

[$CdSO_4$] in the substrate =0 $mg.kg^{-1}$

	Beginning	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
1	0.4138	0.3723	0.4149	0.3821	0.3904	0.3767	0.3752	0.371	0.3589
2	0.5252	0.544	0.4529	0.562	0.5338	0.4513	0.4618	0.4088	0.3958
3	0.5285	0.563	0.6039	0.5814	0.5471	0.476	0.4838	0.4808	0.4332
4	0.5303	0.5808	0.6088	0.5824	0.5694	0.5042	0.5065	0.4882	0.4512
5	0.5515	0.5821	0.615	0.5866	0.6007	0.5173	0.5089	0.489	0.4566
6	0.5536	0.6034	0.617	0.5886	0.6073	0.5225	0.51	0.4983	0.4629
7	0.5541	0.6098	0.6398	0.5893	0.6194	0.5256	0.525	0.5128	0.463
8	0.5724	0.6142	0.6408	0.6135	0.6278	0.5295	0.5456	0.5176	0.4726
9	0.6217	0.6179	0.6475	0.626	0.6355	0.5652	0.562	0.5524	0.4928
10	0.6252	0.628	0.6714	0.6397	0.6648	0.5776	0.6038	0.5643	0.5087
11	0.6442	0.641	0.6791	0.6486	0.6661	0.5883	0.6148	0.5854	0.577
12	0.6486	0.662	0.7251	0.7113	0.6672	0.6227	0.6284	0.6026	0.58
13	0.6761	0.6726	0.7744	0.733	0.7442	0.6582	0.6625	0.6118	0.5826
14	0.6823	0.6891	0.7751	0.7719	0.7804	0.7175	0.7054	0.6694	0.6022
15	0.6914	0.7052	0.7906	0.8149	0.7943	0.7333	0.7389	0.6759	0.6878
16	0.7688	0.7176	0.8167	0.8233	0.8371	0.7499	0.7545	0.7318	0.6894
17	0.8056	0.8604	0.9208	0.841	0.8522	0.7684	0.7761	0.7449	0.6982
18	0.8242	0.9038	0.9601	0.8686	0.8952	0.784	0.8001	0.7686	0.7408
19	0.9136	0.9057	0.9701	0.9189	0.9288	0.7843	0.816	0.7702	0.7577
20	1.0839	1.0799	1.1451	1.056	1.0819	0.9621	0.9772	0.9155	0.912

AVG	0.66075	0.67764	0.723455	0.696955	0.70218	0.62073	0.627825	0.597965	0.56617
STDEV	0.156217	0.156506	0.177	0.15599	0.161762	0.144801	0.149037	0.138119	0.143216

Cd_{PRE}-worms

[$CdSO_4$] in the substrate =600 $mg.kg^{-1}$

	Beginning	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
1	0.4805	0.4577	0.4565	0.4328	0.4459	0.3766	0.2482	0.2082	0.1797
2	0.5764	0.5732	0.5887	0.5595	0.5352	0.5483	0.4584	0.4356	0.3376
3	0.5781	0.6166	0.6067	0.5704	0.6355	0.5891	0.5279	0.5524	0.5151
4	0.5866	0.6309	0.6171	0.6137	0.6479	0.6003	0.5494	0.5878	0.5579
5	0.606	0.6413	0.663	0.634	0.6792	0.7078	0.6476	0.677	0.5632
6	0.6156	0.6472	0.6685	0.6675	0.7237	0.7119	0.6487	0.7092	0.687
7	0.6342	0.6537	0.6868	0.6676	0.7484	0.7251	0.6638	0.7097	0.6929
8	0.6411	0.6752	0.7054	0.6763	0.7536	0.7297	0.7006	0.7202	0.7095
9	0.6479	0.6775	0.7086	0.7223	0.7673	0.7401	0.7397	0.7263	0.7106
10	0.672	0.6925	0.7421	0.7233	0.7854	0.7619	0.7558	0.7325	0.7353
11	0.6863	0.7315	0.7485	0.749	0.7891	0.769	0.7741	0.8248	0.8142
12	0.6928	0.7316	0.7505	0.7543	0.8064	0.779	0.787	0.8389	0.8222
13	0.7035	0.7541	0.753	0.7599	0.8152	0.8355	0.8046	0.841	0.8311
14	0.7195	0.7623	0.7648	0.7645	0.8491	0.8488	0.8102	0.8956	0.8405
15	0.7818	0.782	0.8149	0.8062	0.8696	0.8713	0.8184	0.9794	0.8578
16	0.8095	0.8097	0.8262	0.824	0.889	0.901	0.8898	0.9848	0.9206
17	0.8202	0.8172	0.8854	0.8263	0.893	1.0367	0.9817	1.0003	0.9544
18	0.8408	0.8394	0.8879	0.8553	1.0553	1.0551	0.9876	1.0298	0.9772
19	0.9212	1.0153	0.9709	0.925	1.0554	1.0721	1.0182	1.0458	1.0307
20	0.9861	1.0433	0.9942	0.9829	1.1056	1.0818	1.0859	1.2046	1.0623

AVG	0.700005	0.72761	0.741985	0.72574	0.79249	0.787055	0.74488	0.785195	0.73999
STDEV	0.126242	0.137424	0.131203	0.129913	0.165291	0.185013	0.203174	0.232739	0.225421

Cd_{PRE}-worms

[$CdSO_4$] in the substrate =1200 $mg.kg^{-1}$

	Beginning	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
1	0.4718	0.5078	0.4412	0.3521	0.3293	0.261	0.2548	0.2137	0.1985
2	0.5213	0.5188	0.4552	0.3595	0.418	0.3677	0.3225	0.3282	0.2981
3	0.5307	0.5197	0.4634	0.3709	0.4366	0.4205	0.4199	0.3613	0.3462
4	0.5473	0.5398	0.4658	0.3733	0.4512	0.4375	0.4209	0.3875	0.3498
5	0.554	0.543	0.4687	0.3887	0.4653	0.4676	0.4441	0.3968	0.3513
6	0.5641	0.5465	0.4807	0.4064	0.4956	0.4682	0.4536	0.4013	0.3787
7	0.6394	0.575	0.4926	0.4175	0.497	0.4816	0.4615	0.4245	0.3984
8	0.649	0.6418	0.5496	0.4607	0.5144	0.4836	0.4898	0.4269	0.409
9	0.6713	0.653	0.5802	0.4727	0.5182	0.5034	0.4917	0.4356	0.4347
10	0.6846	0.6651	0.584	0.4862	0.5218	0.5039	0.5066	0.4644	0.4452
11	0.6985	0.6993	0.5967	0.4984	0.5623	0.5233	0.5121	0.4824	0.4521
12	0.7124	0.7166	0.597	0.5037	0.5844	0.5498	0.5287	0.5003	0.4603
13	0.7262	0.7243	0.6015	0.5283	0.6275	0.575	0.602	0.525	0.4911
14	0.7516	0.7252	0.6376	0.5375	0.6384	0.6167	0.6158	0.5614	0.5076
15	0.757	0.731	0.6488	0.5464	0.6472	0.6202	0.6369	0.5641	0.53
16	0.7931	0.7619	0.6522	0.5621	0.672	0.6378	0.6417	0.5774	0.5547
17	0.815	0.7751	0.6621	0.5796	0.6933	0.6642	0.6417	0.5849	0.5628
18	0.8228	0.8107	0.6735	0.581	0.696	0.6858	0.6464	0.6523	0.6568
19	0.8631	0.8109	0.7509	0.6273	0.7175	0.7533	0.7344	0.677	0.6769
20	0.8686	0.8497	0.7791	0.6993	0.8642	0.7778	0.8048	0.7254	0.7308

AVG	0.68209	0.66576	0.57904	0.48758	0.56751	0.539945	0.531495	0.48452	0.46165
STDEV	0.120404	0.110988	0.100239	0.096992	0.127063	0.128586	0.134634	0.126236	0.132219

Cocoon production, percentage viable cocoons, and the mean number of hatchlings per cocoon

Cd_{ABS}-worms

$[CdSO_4]$ in the substrate:	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Total cocoon production	% Viable cocoons	Mean amount of hatchlings per cocoon
0 $mg.kg^{-1}$	41	81	64	78	38	25	54	29	410	71.91	2.48 ± 1.51
600 $mg.kg^{-1}$	11	28	20	57	46	31	46	8	247	40.74	1.72 ± 1.74
1200 $mg.kg^{-1}$	0	8	7	36	26	13	14	4	108	35.07	1.47 ± 1.08

Cd_{PRE}-worms

$[CdSO_4]$ in the substrate:	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Total cocoon production	% Viable Cocoons	Mean amount of hatchlings per cocoon
0 $mg.kg^{-1}$	14	22	16	58	53	5	1	0	169	85.56	2.58 ± 1.51
600 $mg.kg^{-1}$	38	33	20	53	26	1	19	2	192	64.18	2.03 ± 1.38
1200 $mg.kg^{-1}$	17	12	1	2	11	3	3	2	51	46.51	1.44 ± 1.04

Cellpercentage of coelomic fluid

$[CdSO_4] = 3000\ mg.kg^{-1}$

Control	$^{N}Cd_{PRE}$	$^{B}Cd_{PRE}$	$^{N}Cd_{ABS}$	$^{B}Cd_{ABS}$
6.4	7.1	4.5	7.4	6.4
8.8	13.2	13	6.1	6.4
10.9	6.2	10.1	7	1.3
7.9	7.8	8.5	9.5	8.4
12	5.4	3.2	6.2	9
9.2	7.5	15.1	4.5	2.2
14.4	6.8		11.8	0.7
7.2	17.9		10.6	4.5
8.8	9.6		5.7	
8.5	9.1		10.4	
	16.5		5	
	19.9		11	
	8.14		10	
	9.27		10.1	
	7.9		11.3	
	10.7		10.7	
	9.8		7.3	
	14.5		8.5	
	16.9			
	13.9			
	10.3			
	14.5			

Osmolality of coelomic fluid

$[CdSO_4] = 3000\ mg.kg^{-1}$

Control	$^{N}Cd_{PRE}$	$^{B}Cd_{PRE}$	$^{N}Cd_{ABS}$	$^{B}Cd_{ABS}$
168	176	128	192	153
155	184	155	153	150
169	173	139	177	113
171	189	123	173	163
170	194	125	172	167
166	161	126	165	136
153	163		169	103
168	193		193	104
157	186		162	146
157	200		176	
	175		178	
	189		184	
	184		184	
	230		186	
	156		186	
	158		171	
	171		183	
	159		188	
	169			
	169			
	188			
	195			